1908 The Golden Anniversary 1958
of
The American Phytopathological Society

VOLUME 48 MAY, 1958 NUMBER 5
SECTION 1

Phytopathology

An International Journal

Official Organ of

THE AMERICAN PHYTOPATHOLOGICAL SOCIETY

UNIVERSITY OF MICHIGAN
JUN 1 1958
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CHEMICAL REACTIONS INVOLVED IN THE FUNGITOXICITY OF CAPTAN[1]

Raymond J. Lukens[2] and Hugh D. Sisler

SUMMARY

Studies were conducted to determine the chemical basis of the fungitoxicity of captan (*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide) to *Saccharomyces pastorianus* E. C. Hans. Toxicity of this fungicide increases as the initial pH of the medium is decreased over the range of pH 7.5 to 4.5. The sulfhydryl compounds cysteine, homocysteine, glutathione, coenzyme A, and thioglycerol destroy the fungitoxicity of captan if they are added to yeast cultures simultaneously with or before the fungicide. Cysteine at 3 times the molar concentration of captan protects yeast cells from the toxicity of this fungicide.

Cysteine reacts with captan *in vitro*. The products of this reaction are cystine, tetrahydrophthalimide, hydrogen sulfide, carbon disulfide, 2-thiazolidinethione-4-carboxylic acid, and hydrochloric acid. The trichloromethylthio group and thiophosgene are apparently intermediates in the formation of 2-thiazolidinethione-4-carboxylic acid, since the latter compound could also be formed by reacting trichloromethylsulfenyl chloride or thiophosgene with cysteine. Although captan does not appear capable of reacting with other than sulfhydryl groups, the trichloromethylthio group or thiophosgene released from captan by sulfhydryl groups is apparently capable of reacting with amino, hydroxyl, sulfhydryl, and possibly other groups. Histidine and serine react with thiophosgene to form ultraviolet-absorbing compounds.

Carbon disulfide and hydrogen sulfide are evolved from thiophosgene in aqueous suspensions. These 2 gases are also evolved when captan reacts with cysteine in aqueous or alcoholic solutions. The formation of carbon disulfide from captan or from thiophosgene probably involves a di(thiophosgene) intermediate. A compound with the melting point of di(thiophosgene) was isolated from aqueous solutions containing thiophosgene. Carbon disulfide, but not hydrogen sulfide, is evolved when yeast cells are treated with captan. The release of carbon disulfide from captan in yeast cultures suggests that thiophosgene is formed in the reaction of captan with cellular components.

Trichloromethylsulfenyl chloride is nearly as toxic to cells of *S. pastorianus* as is captan, whereas thiophosgene is somewhat less toxic and tetrahydrophthalimide is virtually nontoxic. This would indicate that the actual toxiphore of the captan molecule is the trichloromethylthio group. Thiophosgene, formed from the trichloromethylthio group, is probably the ultimate toxicant. The function of the imide portion of captan appears to be that of regulating the penetration of the trichloromethylthio toxiphore into cells and of stabilizing this toxiphore so that it reacts only when released by certain sulfhydryl groups.

The combination of thiophosgene with free sulfhydryl, amino, hydroxyl, and possibly carboxyl groups of the cell or a combination of the trichloromethylthio group through the free bond of sulfur to certain vital groups in the cell are considered to be the chemical reactions responsible for the fungitoxicity of captan.

Captan (*N*-(trichloromethylthio)-4-cyclohexene-1, 2-dicarboximide), synthesized by reacting tetrahydrophthalimide and trichloromethylsulfenyl chloride, was introduced by Kittleson in 1952 (8) and has rapidly established itself as one of the more important commercial fungicides. Its structural formula is given in Figure 1, A.

Kittleson (8, 9) reported that various compounds derived by attaching the trichloromethylthio group to the nitrogen position of imides, hydantoins, 2,4-oxazolidinediones, and sulfonamides are fungitoxic. Since the *N*(trichloromethylthio) group is present in all of these compounds, he postulated that it is the active portion of the molecule. Two recent sources support the views of Kittleson. Waeffler et al. (18) have investigated a series of sulfonamide derivatives that contain the *N* trichloromethylthio group, several of which show promise of becoming good fungicides. Van der Kerk (16) reported that Uhlenbroek has disclosed a new group of trichloromethylthio compounds, known as trichloromethylthio sulfonates, possessing a general structure of $R\text{-}SO_2\text{-}SCCl_3$. Changing the R group is reported to have little effect on the fungitoxicity of these compounds.

On the basis of the similarity of the slopes of dosage response curves of captan and cycloheximide (an antifungal antibiotic containing an imide structure), Horsfall (6) suggested that the 2 compounds may act by the same mechanism and that the toxiphore is

$$\begin{matrix} & O & R & O & \\ & \| & | & \| & \\ -C- & N & -C- & R & \end{matrix}$$

probably R-C-N-C-R. More recently, Horsfall and Rich (7) have discussed 5 structural features in the captan molecule that could be involved in its fungitoxicity: ". . . the $-CCl_3$ group, the sulfur bridge, the diketone, the *N*-heterocycle, and the mobile hydrogens on the carbon atoms *alpha* to the ketones." The last feature

[1] Accepted for publication November 14, 1957.

Portion of a thesis submitted to the Graduate School, University of Maryland, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, September, 1957.

Scientific publication No. 650, Contribution No. 2840, of the University of Maryland Agricultural Experiment Station, Department of Botany.

This investigation was supported in part by research grants C-2307(C3) of the National Cancer Institute and E-225(C4) of the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Public Health Service.

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PHYTOPATHOLOGY for April (48: 179-234) was issued May 2, 1958.

was considered of little importance because the phthalimide analogue, which lacks such hydrogens, is active fungicidally.

On comparing the structures of captan and thiuram disulfide, Mässing (12) suggested that the carbon-sulfur bonds may be the site of toxicity for both compounds.

Hochstein and Cox (4) studied certain effects of captan on fungus metabolism and reported that pyruvic acid accumulates in greater quantities in spores of *Fusarium roseum* treated with captan than in untreated spores. Activity of yeast carboxylase was inhibited by captan, but the inhibition was alleviated upon the addition of cocarboxylase. Inhibition of growth of cells by captan was not alleviated by adding thiamine pyrophosphate or thiamine to the cultures. They suggested that captan may inhibit fungus growth by interfering with decarboxylation reactions requiring thiamine pyrophosphate. Dugger et al (3) have reported that yeast hexokinase activity also is inhibited by captan.

Silber (15) reported significant differences between the slopes of the toxicity curves for spore germination of certain phthalimide, hydantoin, or sulfonamide derivatives containing the *N*(trichloromethylthio) group. Differences in ability to inhibit oxygen consumption of spores were not observed for these derivatives, but differences in ability to inhibit yeast carboxylase *in vitro* were observed. Captan was less effective in inhibiting carboxylase than were several of the other compounds tested. On the basis of his results, he was unable to identify the toxiphore of the captan molecule.

The present work was undertaken to clarify further the chemical basis of the fungitoxicity of captan.

MATERIALS AND METHODS.—*Cultural procedures.*—The test organism throughout these studies was *Saccharomyces pastorianus* E. C. Hans. It was grown in a liquid shake culture at 30°C. The composition of the medium was as follows: glucose, 20 g; ammonium sulfate, 3.0 g; magnesium sulfate, 0.25 g; glycine, 1.0 g; monobasic potassium phosphate, 3.0 g; thiamine-HCl, 20 μg; niacin, 20 μg; biotin, 1 μg; *i*-inositol, 200 μg; pyridoxine, 10 μg; folic acid, 10 μg; and distilled water to make 1 liter. The trace element supplement of Burkholder (2) was also included in this medium. The medium was adjusted to pH 6.0 with KOH solution. Duration of these experiments was either 24 or 48 hours when the initial amount of yeast used was 0.058 mg (dry wt.)/ml, and 8–12 hours when 20 times this amount of yeast was used as inoculum. Rapidly growing (24-hour) cultures were used. The desired concentration of captan in these cultures was obtained by adding 0.1-ml aliquots of stock solutions that were prepared in 95 per cent ethanol for each experiment. When compounds were tested as antagonists to the fungitoxicity of captan, sterile stock solutions were prepared by wetting the material with 1 ml of 95 per cent ethanol prior to adding sufficient sterile medium to make a final volume of 100 ml. One ml of appropriate stock solutions of an antagonist was added to each culture. The total volume of a culture was 20 ml. Growth of the yeast was determined by measuring the optical density of the cultures at 450 mμ.

Analytical procedures.—The products of the reaction between captan and cysteine were identified and their concentrations determined by the following procedures. One-tenth millimole of captan in 100 ml of 70 per cent ethanol was reacted with a range of concentrations of cysteine. The concentration and rate of formation of titratable hydrogen ion were determined by base titration with 0.01 N sodium hydroxide. The reacting solution was maintained at pH 6.0 by adding the base when necessary. In the determination of the concentration of chloride ion, the solution was treated with 10 ml of a 2 per cent zinc acetate solution (to remove cysteine and other components that interfere with the silver ion) prior to addition of silver nitrate by the regular Volhard (14) method.

Hydrogen sulfide and carbon disulfide were collected simultaneously by passing air over a stirred reaction-mixture, through a 2 per cent zinc acetate solution to remove hydrogen sulfide, and then through Viles' solution (17) to remove the carbon disulfide. A trap of the last solution was placed in the line entering the reaction mixture to free the air of contaminating sulfur gases before it reached the reaction flask. Zinc sulfide was determined iodimetrically by back-titrating with sodium thiosulfate. The amount of copper diethyldithiocarbamate formed by the reaction of carbon disulfide with the trapping solution was determined spectrophotometrically as 440 mμ.

Paper chromatography and ultraviolet spectrophotometry were used in the isolation and identification of the organic products from the reaction mixture. Ultraviolet absorption spectra were determined with a Beckman DU spectrophotometer. The chromatograms, which were developed in a glass jar, were made by the ascending technique. The solvent was either l-butanol, ethanol, and water (4:1:1 by volume), or l-butanol, acetic acid, and water (80:6:10 by volume). The chromatograms were developed for 18–24 hours and examined under an ultraviolet light to locate absorbing and fluorescing compounds. They were sprayed with a 0.2 per cent ethanolic ninhydrin solution and heated at 100°C for 10 minutes to locate amino acids.

A technique was developed for the identification of tetrahydrophthalimide. The chromatogram was sprayed with a 1:1 (by volume) mixture of 1 N potassium hydroxide and a 5.25 per cent solution of sodium hypochlorite (Clorox) and heated for 15 minutes at 100°C. Following this treatment, 10 μg or less of tetrahydrophthalimide is detectable as a fluorescent spot in ultraviolet light. Apparently, the imide is partially hydrolyzed by the base to the half-amide state, and the resulting compound is oxidized to the corresponding *ortho* amino acid by the sodium hypochlorite. This test may not be specific for tetrahydrophthalimide, but probably would detect other imides as well.

Recrystallized captan, tetrahydrophthalimide, sodium tetrahydrophthalimide, tetrahydrophthallic acid half-amide, and trichloromethylsulfenyl chloride were provided by the California Spray Chemical Company,

TABLE 1.—*Growth of Saccharomyces pastorianus and fungitoxicity of captan in liquid shake cultures adjusted to different initial pH values*

Concentration of captan (ppm)	Yeast growth (optical density)					
	pH 4.5		pH 6.0[a]		pH 7.5[a]	
	24 Hours	48 Hours	24 Hours	48 Hours	24 Hours	48 Hours
0.00	0.86	4.20	0.53	4.80	0.11	5.90
0.01	0.38	4.20	0.37	4.40	0.08	4.70
0.05	0.01	3.70	0.14	4.80	0.05	4.05
0.1	0.00	2.20	0.07	4.60	0.05	4.20
0.5	0.00	0.01	0.01	1.70	0.02	3.40
1.0	0.00	0.01	0.01	0.01	0.01	3.80
5.0	0.00	0.01	0.00	0.01	0.00	0.01

[a] pH was adjusted with KOH solution.

Moorestown, N. J. Thiophosgene was purchased from the Delta Chemical Works, Inc., New York, N. Y.

RESULTS.—In preliminary studies, it was determined that the toxicity of captan to *S. pastorianus* increases as the initial pH of the medium is decreased (Table 1). The rate of growth of untreated cells was most rapid in a medium with an initial pH of 4.5, but the total growth in 48 hours was greatest in the medium with an initial pH of 7.5. The greater yield at the higher pH levels is attributed to the greater buffering capacity of these media for hydrogen ions, which reach inhibitory concentrations earliest in the medium with an initial pH of 4.5. Although captan is somewhat more effective at pH 4.5 than at 6.0, an initial pH of 6.0 was employed in these studies because it is in the range that permits the use of phosphate buffer.

In order to determine the types of cellular components that captan is capable of reacting with, a number of organic compounds, most of which are biochemicals, were tested as antagonists to the fungitoxicity of captan. Included in this group were most of the commonly occuring amino acids, purine and pyrimidine bases and their corresponding nucleosides, vitamins, organic acids, and carbohydrates. A complete list of the chemicals tested and the rating of their effectiveness in antagonizing the toxicity of captan is available (11).

Appreciable antagonistic action against the fungitoxicity of captan was exhibited only by the sulfhydryl compounds cysteine, glutathione, homocysteine, coenzyme A, and thioglycerol. Thioglycollic acid, tyrosine, cytosine, and *p*-phenylenediamine showed slight activity but, except for thioglycollic acid, were not considered to be active enough to warrant further investigation in the present study.

Comparisons were made of the effectiveness of cysteine, thioglycollic acid, and thioglycerol as antagonists of the fungitoxicity of captan. Cysteine and thioglycerol were very effective, whereas thioglycollic acid was a relatively weak antagonist (Table 2). All 3 compounds contain a 3-carbon chain and a sulfhydryl group but differ in other groups attached to the chain. The amino group of cysteine and the hydroxyl groups of thioglycerol (which are lacking in thioglycollic acid) apparently contribute to the activity of these compounds.

These results suggest that an interaction between captan and sulfhydryl groups of cellular components may be involved in the mechanism of toxicity. Because of its high activity, cysteine was chosen as the sulfhydryl compound for further investigation of the captan-sulfhydryl interaction.

To determine whether cysteine acts directly on captan or reverses the action of the fungicide within the yeast cell, cells were exposed to captan before cysteine was added, exposed to captan and cysteine simultaneously, or exposed to a reaction mixture of these chemicals that had been incubated 1 hour before addition to the culture. Cysteine, at the concentration used (3×10^{-4} M), had little effect when added to the yeast culture after 1 hour of incubation with captan, but it was effective in offsetting toxicity when added 1 hour before or simultaneously with captan (Table 3). When the toxic reactions of captan have taken place, it is difficult to reverse them with cysteine. If cysteine reacts directly with captan to destroy its toxic principle,

TABLE 2.—*Comparison of cysteine, thioglycollic acid, and thioglycerol as antagonists of the toxicity of captan to Saccharomyces pastorianus in liquid shake cultures*

Concentration of captan (ppm)	Yeast growth (optical density after 24 hours) when indicated antagonist was added[a]			
	None	Cysteine	Thioglycollic acid	Thioglycerol
0.0	3.72	3.42	3.02	3.68
0.1	0.01	3.48	2.35	3.70
1.0	0.00	3.55	0.08	2.73
5.0	0.00	2.08	0.00	1.50
10.0[b]	0.00	1.61	0.00	0.10

[a] Concentration of antagonists was 3×10^{-4} M.
[b] Captan at 10 ppm is equivalent to 3×10^{-5} moles/liter.

TABLE 3.—*The effectiveness of cysteine in protecting cells of Saccharomyces pastorianus from the toxicity of captan in relation to the order of addition of inoculum, fungicide, and antagonist to the medium*

Order of addition to the medium[a]	Yeast growth[b] (percentage of control)	
	24 Hours	48 Hours
Inoculum + captan + cysteine	74.7	100.4
Inoculum + captan; cysteine 1 hour later	0.0	24.8
Inoculum + cysteine; captan 1 hour later	91.8	97.9
Captan + cysteine added 1 hour before inoculum	89.0	81.9
Inoculum + captan	0.0	6.0
Inoculum + cysteine	96.6	98.1

[a] Items connected by plus signs were added simultaneously. Concentration of captan was 1.7×10^{-5} M, and that of cysteine was 3×10^{-4} M.
[b] Data are the mean percentage of 4 experiments.

TABLE 4.—*Toxicity of captan to Saccharomyces pastorianus in liquid shake cultures containing various concentrations of cysteine*

Captan:cysteine[a] molar ratio	Yeast growth[b] (percentage of control after 8 hours)	
	Captan 1 ppm	Captan 5 ppm
1:0.00	19	1
1:0.25	16	0
1:0.5	38	67
1:1	60	93
1:2	84	88
1:3	95	95

[a] Captan was added in 95 per cent ethanol. The final concentration of the solvent in the culture was 0.5 per cent.
[b] Mean of 3 experiments.

antagonism would be expected at low molar ratios. Data in Table 4 shows that 1–3 molar equivalents of cysteine are sufficient to protect the yeast cells from the toxic effects of captan when the concentration of the fungicide is 1–5 ppm. The results of these experiments indicate that a direct reaction occurs between captan and sulfhydryl compounds. Attention was therefore turned to a study of the reaction of captan and sulfhydryl compounds *in vitro* as a possible model of similar reactions that may occur in cells.

When captan and cysteine are mixed in aqueous or 70 per cent ethanolic solutions, the pH decreases markedly and chloride ions are released. In mixtures buffered at pH 6.0, a white precipitate is formed. The production of titratable hydrogen ion in this reaction is rapid, nearing completion in 5 minutes. For each mole of captan, slightly in excess of 1 mole of hydrogen ion is produced per mole of cysteine added until 3 moles of cysteine are used (Fig. 2). Further additions of the sulfhydryl compound result in no appreciable increase in the amount of titratable hydrogen ion produced. The amount of chloride ion released per mole of captan is approximately equivalent to the amount of cysteine added until a maximum of 3 moles of cysteine are used (Table 5).

The white precipitate that is formed in this reaction was shown to be cystine. The thermal decomposition point was similar to that reported for cystine (5), and it gave a positive ninhydrin test, a positive nitroprusside test in the presence of sodium cyanide, and a negative test in the absence of the cyanide reagent. The R_f value and the sulfur content were the same as

TABLE 5.—*Release of chloride ion in the reaction of captan with cysteine in 70 per cent ethanol buffered at pH 6.0 with 0.1 M acetate buffer*

Captan[a]:cysteine molar ratio	Moles of chloride ions per mole of captan in 1 hour[b]
1:0	0.27
1:1	1.04
1:2	2.06
1:3	2.92
1:4	3.22

[a] One-tenth millimole of captan was used in each sample.
[b] Mean of 3 experiments, each of which consisted of 3 titrations.

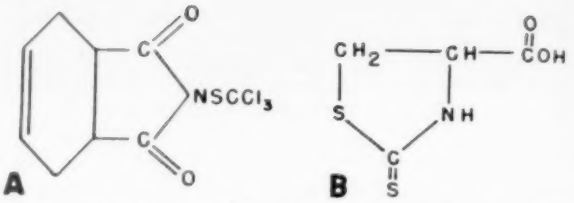


FIG. 1. A) Structural formula of captan. B) Structural formula of 2-thiazolidinethione-4-carboxylic acid.

those of cystine. The yield of cystine would account for approximately $^2/_3$ of the weight of cysteine used in the reaction.

During the course of the reaction between captan and cysteine, several gases are produced. Immediately after the addition of cysteine to a captan solution, a gas is evolved, the odor of which is similar to that of commercial captan formulations. This odor is the same as that of thiophosgene (Cl-C(=S)-Cl), and it was assumed that thiophosgene escapes from the reaction mixture. Several minutes after the beginning of the reaction the odor of hydrogen sulfide was present, and approximately ½ hour later the odor of carbon disulfide also could be detected. Hydrogen sulfide and carbon disulfide were collected simultaneously from the vessel in which captan and cysteine (0.1:0.3 millimole) were reacted in a solution at pH 6.0. In a reaction of 6 hours' duration, 0.01 millimole of hydrogen sulfide and 0.01 millimole carbon disulfide were produced. The sulfur in the hydrogen sulfide and carbon disulfide would account for 30 per cent of the sulfur of the captan in the reaction. Captan alone or cysteine

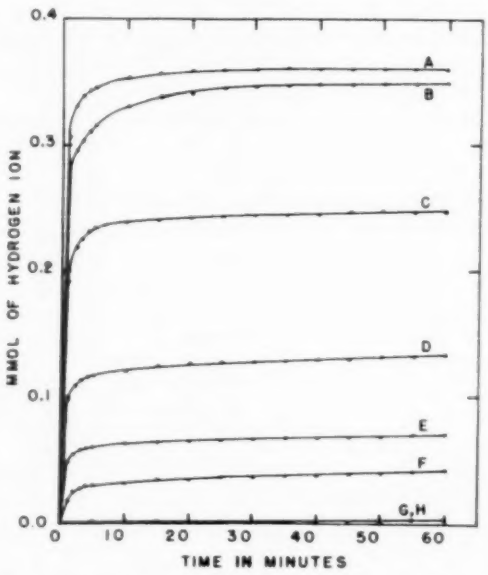


FIG. 2. Acid produced in the reaction of 0.1 millimole of captan with various amounts of cysteine in a 70 per cent ethanolic solution maintained at pH 6.0. Millimoles of cysteine were as follows: A) 0.4; B) 0.3; C) 0.2; D) 0.1; E) 0.05; F) 0.025; G) 0.4 (but no captan); H) none.

alone did not produce these gases under the same conditions. The formation of these gases will be considered in more detail later in this paper.

Further studies of the reaction between captan and the sulfhydryl compounds were made by ultraviolet spectrophotometry and paper chromatography. Upon the mixing of captan and cysteine in water or in ethanol (30 or 70 per cent), a compound is formed that has an absorption maximum at 272 $m\mu$ (Fig. 3). This compound is not cystine, tetrahydrophthalimide, tetrahydrophthalic acid half-amide, or trichloromethylsulfenyl chloride, since these compounds do not absorb strongly at this wave length. Solutions of the products of the reactions between captan and sulfhydryl compounds (thioglycollic acid, thioglycerol, thiourea, homocysteine, coenzyme A and glutathione) were examined in the ultraviolet spectrophotometer. Only thioglycerol appears to be capable of forming an ultraviolet-absorbing compound comparable to that formed with cysteine. The maximum absorption by this compound is at 282–284 $m\mu$.

The organic products of the reaction between captan and cysteine were separated by paper chromatography. Examination of the paper under short-wave ultraviolet light revealed a single dark purple spot. The ultraviolet absorption spectrum of this compound matched that of the compound illustrated in Figure 3. When the paper was sprayed with ninhydrin, spots corresponding in R_f values to cysteine and to cystine were detected. Tetrahydrophthalimide was located as a fluorescing spot under ultraviolet light following treatment of the papers with an alkaline solution of sodium hypochlorite. Traces of *o*-aminotetrahydrophthalic acid apparently were produced, since a small fluorescing spot corresponding in R_f value to an oxidized sample of the half amide of tetrahydrophthalic acid was always present.

When captan is completely decomposed by cysteine, essentially all of the tetrahydrophthalimide originally present in this compound could be recovered from aqueous solutions by successive extractions at pH 6.0 with ethyl acetate. The recovered material had a chromatographic R_f value and a melting point (134°C) comparable to those of a known sample of tetrahydrophthalimide.

Further studies of the nature of the unidentified ultraviolet-absorbing component were undertaken, since the evidence indicated that this combination product between captan and cysteine might represent a type of toxic combination that could occur in fungus cells. To determine which portion of the captan molecule is involved in the formation of this compound, tetrahydrophthalimide and trichloromethylsulfenyl chloride ($ClSCCl_3$) were mixed separately with cysteine. A compound with the same ultraviolet absorption spectrum and R_f value as those of the compound formed in the reaction between captan and cysteine was found in the reaction mixture of cysteine and trichloromethylsulfenyl chloride. No such component was found in the mixture of cysteine and tetrahydrophthalimide. It is apparent that the formation of this compound involves the trichloromethylthio group of captan and the amino acid, cysteine.

Orwoll (13) has patented a procedure in which trichloromethylsulfenyl chloride is reduced to thiophosgene: In the process, trichloromethylsulfenyl chloride is added to an aryl nucleus containing a replaceable hydrogen atom in the presence of a Friedel-Crafts catalyst. Upon thermal decomposition, thiophosgene is liberated. He suggested that at low temperatures an aryl trichloromethylthio derivative ($R\text{-}S\text{-}CCl_3$) may be formed as an intermediate. It is possible that captan, having a structure similar to this intermediate, may undergo a similar decomposition and release thiophosgene when reacted with sulfhydryl compounds under physiological conditions. Evidence that this type of breakdown of captan does occur was obtained by reacting cysteine and thiophosgene. From the reaction mixture, a product was isolated that had an ultraviolet absorption spectrum and an R_f value identical with those of the ultraviolet-absorbing compound formed in the reaction between cysteine and captan. It is apparent that the ultraviolet-absorbing compound produced in the latter reaction is formed from cysteine and the thiophosgene produced from captan.

It has been reported (1) that phosgene is capable of reacting with the sulfhydryl and amino groups of cysteine to form a 5-membered ring, 2-ketothiazolidine-4-carboxylic acid. If thiophosgene behaves similarly to phosgene, the corresponding thio derivative would be formed when thiophosgene is reacted with this amino acid. The ultraviolet-absorbing compound that is formed by reacting cysteine with captan was shown to be 2-thiazolidinethione-4-carboxylic acid (Fig. 1, B). The method of purification and evidence for the structure of this compound is reported elsewhere (11). 2-thiazolidinethione-4-carboxylic acid and the partially purified compounds obtained from the reaction of cysteine with trichloromethylsulfenyl chloride or with thiophosgene had identical ultraviolet absorption maxima (Fig. 4).

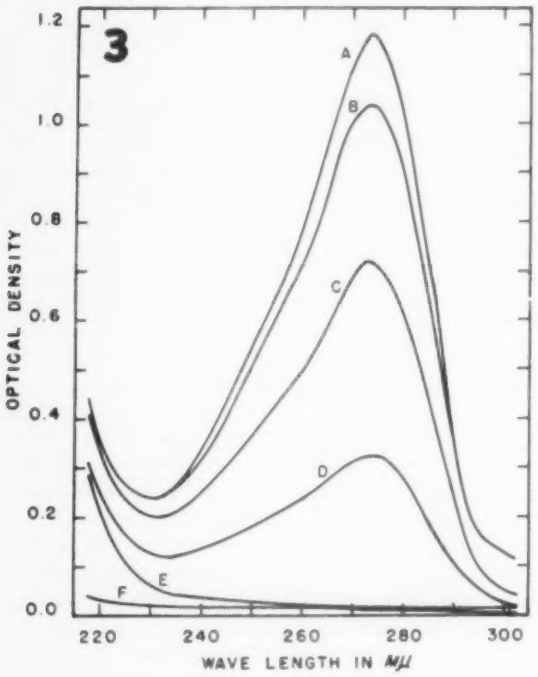
When 2-thiazolidinethione-4-carboxylic acid is formed in a reaction between cysteine and captan, the thione sulfur undoubtedly is derived from the latter compound and would account for 60 per cent of the sulfur originally present in the captan used in the reaction. Thirty per cent of the sulfur is accounted for in the hydrogen sulfide and carbon disulfide that are released. The remaining 10 per cent of the sulfur probably is lost in the thiophosgene that escapes from the reaction mixture.

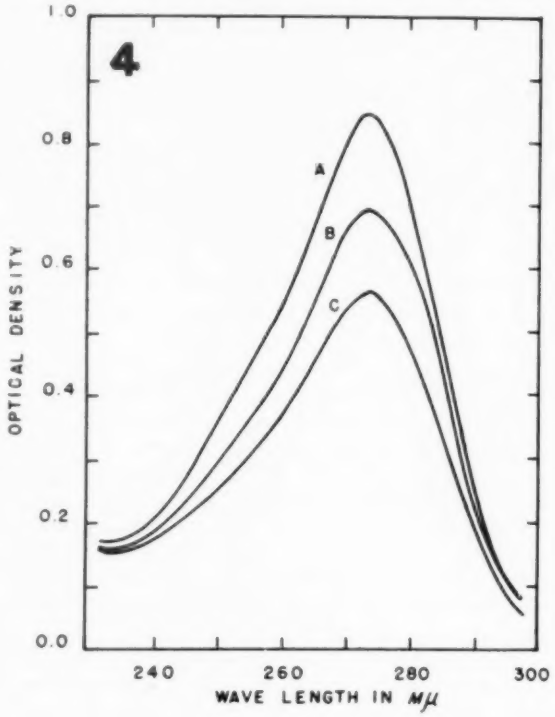
It was possible to show by ultraviolet absorption that thiophosgene and the thiophosgene released from captan by cysteine apparently react with serine to form an ultraviolet-absorbing compound (Fig. 5, 6, 7). A similar compound is also formed, in trace amounts, upon prolonged incubation of captan in aqueous solution with serine. This would indicate that thiophosgene is released when captan decomposes spontaneously in aqueous solution.

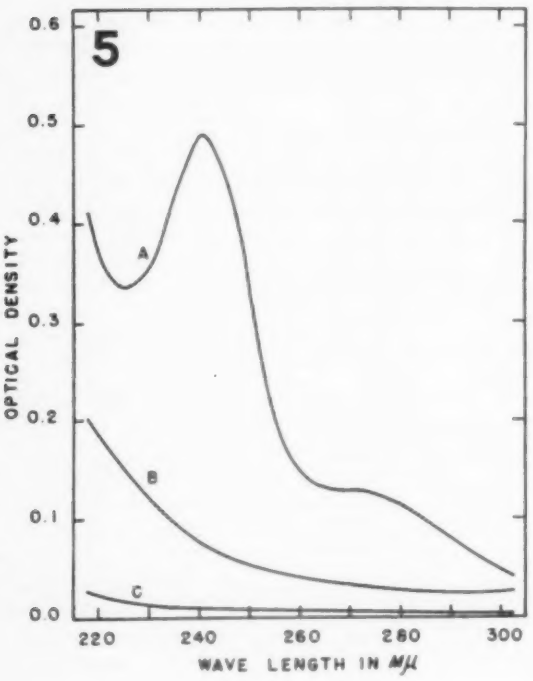
Histidine also forms an ultraviolet-absorbing compound (maximum 270 $m\mu$) when reacted with thio-

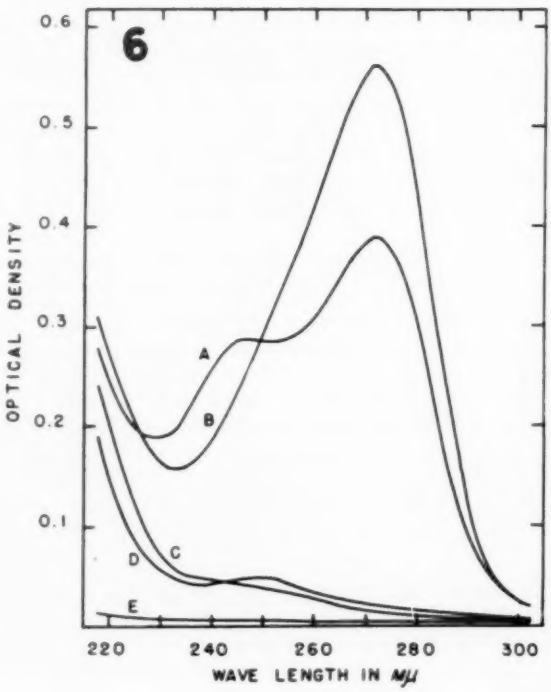
phosgene (Fig. 8) but not when reacted with captan in the presence of cysteine. When the latter reaction mixture was chromatographed, the only ultraviolet-absorbing compound was 2-thiazolidinethione-4-carboxylic acid. Apparently, histidine cannot compete successfully with cysteine for the thiophosgene produced. The possibility that certain other amino acids may also behave like serine and histidine has not yet been investigated.

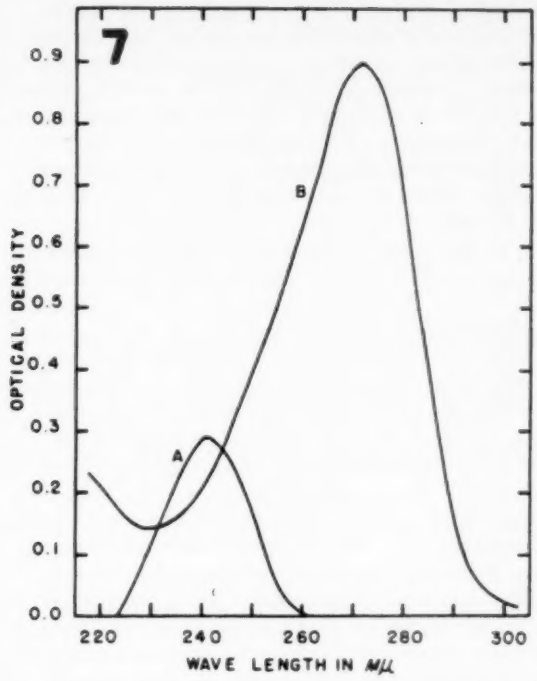
It has been mentioned that hydrogen sulfide and carbon disulfide arise from the reaction of captan and cysteine. These gases also arise from thiophosgene when it is suspended in water. Hydrogen sulfide apparently arises from the reaction between thiophos-











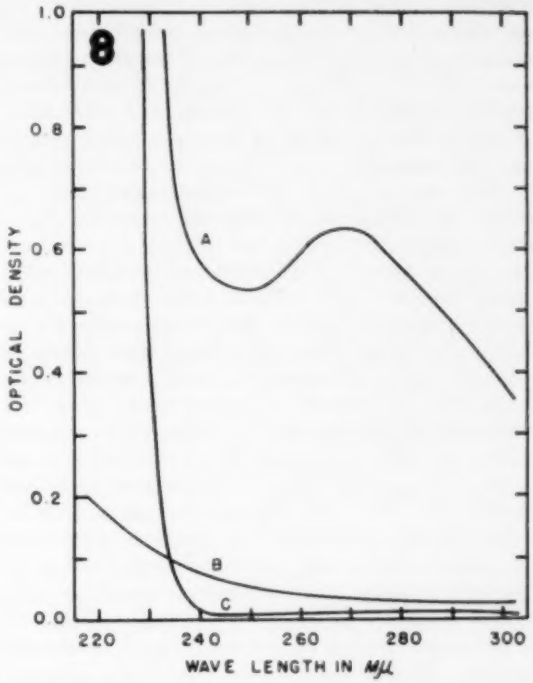


FIG. 7-8. FIG. 7. Ultraviolet absorption spectra of compounds formed in a mixture of captan, cysteine, and serine. The compounds were separated chromatographically with a solvent mixture of 1-butanol, ethanol, and water (4:1:1 by volume) and eluted from the paper with water. A) Compound with an R_f value of 0.10 (identical with reaction product of thiophosgene and serine), and B) compound with an R_f value of 0.20 (2-thiazolidinethione-4-carboxylic acid). (Compare with Fig. 5, curve A.)—FIG. 8. Ultraviolet absorption spectrum A) of compounds in a solution in which thiophosgene was reacted with histidine, B) of thiophosgene, and C) of histidine. Thiophosgene, 0.5 millimole, was reacted with histidine, 0.5 millimole, in 15 ml of 0.1 M phosphate buffer at pH 7.5, and the solution was diluted 1:33 with water before absorption measurements were made.

gene and water molecules, whereas carbon disulfide may be accounted for by decomposition of the di(thiophosgene), $Cl\text{-}\overset{\overset{S}{\|}}{C}\text{-}S\text{-}CCl_3$, that is formed by the condensation of thiophosgene molecules. From an aqueous suspension of thiophosgene, a precipitate that has a melting point of 115°C was obtained; this is in the range reported in the literature for di(thiophosgene) (10). It has been reported that di(thiophosgene) can be produced also by irradiation of thiophosgene with ultraviolet light (10).

When yeast cells were treated with captan, the carbon disulfide released accounted for 30 per cent of the sulfur in the added captan. No hydrogen sulfide was released from the cultures. Carbon disulfide could not be detected over cultures of untreated yeast or over solutions of captan. Evolution of carbon disulfide from yeast treated with captan indicates that thiophosgene is formed in a reaction of this fungicide with components of the fungus cells.

←

FIG. 3-6. FIG. 3. Ultraviolet absorption spectra of a compound formed when 0.1 millimole of captan was reacted with various amounts of cysteine in 100 ml of 70 per cent ethanol maintained at pH 6.0 with 0.1 M phosphate buffer. The solutions were diluted 1:10 with 70 per cent ethanol before ultraviolet absorption measurements were made. Millimoles of cysteine were as follows: A) 0.4; B) 0.3; C) 0.2; D) 0.1; E) none; F) 0.4 (but no captan).—FIG. 4. Comparison of the ultraviolet absorption spectrum of 2-thiazolidinethione-4-carboxylic acid with spectra of compounds produced by reacting cysteine with thiophosgene or trichloromethylsulfenyl chloride in 0.1 M phosphate buffer at pH 6.0. In each case the concentration of sample was 10 ppm. A) 2-thiazolidinethione-4-carboxylic acid. B) Partially purified reaction product of cysteine and thiophosgene. C) Partially purified reaction product of cysteine and trichloromethylsulfenyl chloride.—FIG. 5. Ultraviolet absorption spectrum A) of compounds formed in the reaction of thiophosgene (0.5 millimole) with serine (0.5 millimole) in 15 ml of 0.1 M phosphate buffer at pH 7.5 (diluted 1:33 with water before measurement); B) of thiophosgene; and C) of serine.—FIG. 6. Ultraviolet absorption spectrum of A) compounds in a solution in which captan was reacted with cysteine in the presence of serine, B) of 2-thiazolidinethione-4-carboxylic acid formed by reacting captan and cysteine, C) of captan plus serine, D) of cysteine plus serine, and E) of serine. Captan, 0.1 millimole, and serine, 0.3 millimole were dissolved in 100 ml 30 per cent ethanol buffered at pH 6.0 with 0.1 M acetate buffer. Cysteine, 0.2 millimole, was added to the stirred reaction mixture. The solutions were diluted 1:10 with 30 per cent ethanol before absorption measurements were made.

Whereas trichloromethylsulfenyl chloride may undergo conversion to thiophosgene, which can react with certain amino acids, it may, in certain instances, combine directly through sulfur to form trichloromethylthio derivatives of amino acids and other cellular components without going through a thiophosgene stage. The possibility that this type of combination may occur has not been investigated thoroughly, but it could be an important mechanism in the fungitoxicity of captan.

One would expect thiophosgene or trichloromethylsulfenyl chloride to be toxic to fungi if reactions of the type just described for these compounds are involved in the fungitoxicity of captan. The toxicity of these compounds in comparison with that of captan and tetrahydrophthalimide was determined (Table 6). Trichloromethylsulfenyl chloride was almost as toxic as captan to cells of *S. pastorianus*, thiophosgene was somewhat less so, and tetrahydrophthalimide was only slightly toxic. Thiophosgene was not highly toxic unless glycine was omitted from the medium; apparently thiophosgene reacts with and is neutralized by this amino acid. Captan and trichloromethylsulfenyl chloride were not affected by glycine in the medium. Di(thiophosgene), which is formed from thiophosgene in aqueous solutions, has been reported by Klöpping (10) to have considerable fungicidal activity toward *Botrytis cinerea* Pers. and *Penicillium italicum* Wehm.

Discussion.—The initial action of cysteine on captan appears to be that of splitting the captan molecule at the nitrogen-sulfur bond. This might be considered an oxidation-reduction reaction in which the nitrogen atom of the imide and the trichloromethylthio group are reduced while cysteine is oxidized to cystine. The initial reaction is illustrated as follows:

Captan + 2 cysteine → tetrahydrophthalimide + cystine + HCl + thiophosgene

In the reaction illustrated, only ½ mole each of chloride and of hydrogen ions should be produced per mole of cysteine, but actually more than 1 mole of titratable hydrogen ion and approximately 1 mole of chloride ion are produced for each mole of cysteine. In addition, some 2-thiazolidinethione-4-carboxylic acid is formed from each successive mole of cysteine that is added. Thus, the reaction apparently does not go to completion in the manner illustrated, but the thiophosgene formed must immediately undergo reactions to produce more than the anticipated amounts of acid and chloride ion. The reactions of thiophosgene occur simultaneously with the reaction between cysteine and captan. The fate of thiophosgene may be illustrated as follows:

0.6 Thiophosgene + cysteine → 0.6 2-thiazolidinethione-4-carboxylic acid + 1.2 HCl

0.1 Thiophosgene + 0.2 H_2O → 0.1 H_2S + 0.1 CO_2 + 0.2 HCl

0.2 Thiophosgene $\xrightarrow{H_2O}$ 0.1 di(thiophosgene) → 0.1 CS_2 + 0.1 CO_2 + 0.4 HCl

0.1 Thiophosgene → Escaped as a gas from the reaction mixture.

Some of the cysteine (0.4 mole) apparently remains unreacted. The proportion of thiophosgene going in any direction is probably determined by the conditions of the reaction. If the reaction of captan with cysteine were carried out in a closed system and in solvents inert to thiophosgene, all of the thiophosgene produced would probably be consumed in the formation of 2-thiazolidinethione-4-carboxylic acid.

The titratable acid expected from a reaction of 1 mole of captan with 3 moles of cysteine would be 3.4 moles (2.8 moles of HCl plus 0.6 mole of 2-thiazolidinethione-4-carboxylic acid). Since the pKa of tetrahydrophthalimide is in the vicinity of 9.0, the hydrogen on the imide nitrogen is not titratable at pH 6.0. The amount of acid produced as determined by base titration totaled 3.5 moles. Under similar conditions but with thioglycerol replacing cysteine in the reaction, 1 less mole of hydrogen ion is obtained from a 1:3 molar ratio of captan and thioglycerol.

Although the reactivity of thiophosgene with amino acids has not been investigated extensively, thiophosgene apparently reacts with amino acids other than cysteine, e.g. glycine, serine, and histidine. Cysteine forms a 2-thiazolidinethione ring. It is likely that a similar ring structure is formed with serine but to a lesser extent; however, serine may form a thiourea derivative to an appreciable degree, since the major component that is formed upon reacting serine and thiophosgene absorbs at 240 mμ, the region of maximum absorption of thiourea and –SH groups. When the α-amino group of cysteine is in a peptide bond, as it is in the case of glutathione and coenzyme A, the amide hydrogen is apparently not readily replaced, since no spectrophotometric evidence was obtained for the formation of a thiazolidinethione ring when captan was reacted with these sulfhydryl compounds. A straight-chain compound involving only the sulfhydryl group probably is formed instead. In the case of the reaction between pyruvic acid and cysteine or glutathione, it has been reported (1) that a thiazolidine ring is formed in the case of cysteine and a semimercaptal is formed in the case of glutathione.

Table 6.—*Growth of Saccharomyces pastorianus in liquid shake cultures containing various concentrations of captan, trichloromethylsulfenyl chloride, thiophosgene, or tetrahydrophthalimide*

Concentration of toxicant (ppm)*	Yeast growth (optical density after 24 hours)			
	Captan	Trichloromethylsulfenyl chloride	Thiophosgene	Tetrahydrophthalimide
0.0	2.84	2.84	2.84	2.84
0.1	0.19	0.42		
1.0	0.01	0.01	2.89	2.19
10.0	0.01	0.01	0.02	2.43
100.0		0.01	0.01	2.30

* Captan and tetrahydrophthalimide were added as 95 per cent ethanolic solutions; trichloromethylsulfenyl chloride and thiophosgene were added as anhydrous ethyl ether solutions. The final concentration of ethanol or ether in the cultures was 0.5 per cent.

The reaction of captan with cysteine may illustrate a chemical basis for toxicity of captan to fungus cells. The capacity of captan to oxidize sulfhydryl groups does not appear to be the principal basis of its toxicity. This type of inhibition would probably be rendered ineffective by the cell's own reducing mechanisms and by the addition of sulfhydryl compounds. Oxidation of sulfhydryl groups cannot be completely discounted as a possible toxic mechanism. Physical damage to the cell might result from this type of reaction the effects of which cannot be repaired by the cell or by the addition of sulfhydryl compounds.

Although the reactions in which sulfhydryl groups are oxidized by captan are not considered to be directly concerned in toxicity, they provide a mechanism by which the toxic principle of captan is released for action. The possibility that other chemical groups may be capable of releasing the trichloromethylthio group from the captan has not been investigated. Spontaneous release of this group does not appear to be rapid enough to account for the toxicity of captan. The reactions that appear capable of accounting for the toxicity of captan are the combination of thiophosgene with free sulfhydryl, amino, hydroxyl, and possibly carboxyl groups, or a combination of the trichloromethylthio group through the free bond of sulfur to certain groups on enzymes, coenzymes, or other vital cellular components. Thiophosgene probably makes toxic combinations in the cell through formation of a

bridge between 2 properly spaced reactive groups. Cellular components would be linked to the carbon of thiophosgene in this type of bridge. Bridging could occur between adjacent protein or nucleic acid chains, between substrate and enzyme, between enzyme and coenzyme, or between other types of cellular components. Bridging within a protein chain to form ring structures such as 2-thiazolidinethione would likely occur if cysteine or serine were the terminal amino acids. A combination of thiophosgene with a single sulfhydryl group by an alkylation type of reaction also may be toxic.

Because of the high reactivity of captan with sulfhydryl groups, sulfhydryl-dependent enzymes should be expected to be among the most sensitive to this fungicide. The 2 enzymes thus far reported to be inhibited by captan are sulfhydryl-dependent enzymes (3, 4); however, the inhibition of these enzymes by captan may not necessarily involve the sulfhydryl groups.

The data in Table 6 indicate that the trichloromethylthio portion of captan can account for essentially all of the toxicity of this fungicide to *S. pastorianus*. Although the linkage of the trichloromethylthio group through sulfur to vital cellular components may account for the toxicity of this group, thiophosgene produced intracellularly from this group is probably the ultimate toxicant of captan. Several factors can readily account for the fact that the toxicity of thiophosgene to yeast is lower than that of trichloromethylsulfenyl chloride. Thiophosgene is more volatile than is trichloromethylsulfenyl chloride and would thus be lost from the cultures more rapidly; it is probably more reactive with water and with nonvital constituents of the cell, and it may not penetrate to vital sites as readily as does the trichloromethylsulfenyl chloride. Trichloromethylsulfenyl chloride undoubtedly combines with reactive groups in the cell through the sulfur atom, forming trichloromethylthio derivatives. This combination is probably not the most toxic one since cysteine or glutathione probably can free the trichloromethylthio group as they do in the case of captan. Release of carbon disulfide from yeast cultures treated with captan suggests that thiophosgene is actually formed from captan. It is evident that carbon disulfide cannot come directly from trichloromethylsulfenyl chloride or from captan but must go through a thiophosgene or some similar intermediate that undergoes condensation to the di(thiophosgene) form and subsequently decomposes to release the gas.

The function of the imide portion of captan appears to be that of stabilizing the trichloromethythio toxiphore and of modifying the penetration characteristics and reactivity of this group. Thus captan should possess specificity not found in trichloromethylsulfenyl chloride or in thiophosgene.

It would appear that the action of this fungicide, although more specific than that of thiophosgene or trichloromethylsulfenyl chloride, should be somewhat general in nature. Both the specific and general nature of captan as a toxicant is illustrated by the fact that it inhibits yeast growth and is toxic to bean and tomato roots when the concentration of the fungicide in the liquid culture is 1 ppm whereas it is nontoxic to the foliage of higher plants at concentrations of 2000 ppm. The toxicity of captan to the roots of tomato and bean plants in liquid culture has been reported by Silber (15) and has been confirmed by this laboratory (unpublished data). This would suggest a specificity for captan that seems to be regulated by morphological or physiological features of the cell that affect the penetration of the toxicant. In the case of higher plants, it is probable that the cuticle layer protects the leaf cells from captan, whereas the root cells, lacking this protective layer, are susceptible to the fungicide.

The data presented in this paper indicate that the trichloromethylthio group attaching to vital cellular components through sulfur or acting through a thiophosgene intermediate can account for the fungitoxicity of captan to *S. pastorianus*.

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EFFECT OF HIGH TEMPERATURE ON SITE AND EXTENT OF MULTIPLICATION OF SOUTHERN BEAN MOSAIC VIRUS[1]

I. R. Schneider and J. F. Worley[2]

SUMMARY

High temperature (32°C) immediately after inoculation of detached bean leaves with southern bean mosaic virus induced necrosis of tissues primarily in veins rather than between veins. As the length of exposure to high temperature increased, necrosis of tissues in the veins increased and the occurrence of isolated local lesions between the veins decreased. The amount of vein necrosis in relation to the number of local lesions also depended upon whether the start of high-temperature exposure after inoculation was delayed 24, 48, or 72 hours. With a delay of 72 hours only local lesions occurred, even when 72 hours of exposure to high temperature followed this delay.

High temperature before inoculation of detached leaves induced only a small amount of vein necrosis, but it induced an increase in the average size and number of local lesions.

A much larger amount of virus was detected in necrotic areas than in the adjacent non-necrotic regions when the necrosis was associated with either local lesions or veins. Necrotic symptoms apparently indicated the regions in which most virus was present.

Much more virus was detected in samples from leaves exposed to 32°C after inoculation than in samples from leaves exposed to 21°. The difference increased as the duration of the high-temperature period was lengthened. Maximum virus multiplication occurred in the interveinal areas when leaves were exposed to low temperature following inoculation but in the veinal areas with high-temperature exposure.

Temperature has a marked effect on virus multiplication (2, 3, 5, 6, 10, 11, 12, 13, 19). Temperature, particularly high temperature, also affects infection and lesion formation. Investigations on infection and on lesion formation included a wide variety of methods and environmental conditions, such as differences in temperature, in period of exposure, in the relation of exposure to the time of inoculation, and in the virus-host combinations. These different approaches to the problem and the differences in virus-host combinations studied may account for the variety of results reported. Responses to high-temperature treatments include prevention of infection (1), prevention of lesion production or reduction of number of lesions (6), increase in number of lesions (4, 7, 9, 20, 21, 22), increase in lesion size (21, 22), and alteration in type of symptom (15, 18).

The effects of high temperature (32°C) on site and extent of southern bean mosaic virus (SBMV) multiplication were investigated. The results have been reported briefly (16).

Materials and general methods.—Two bean (*Phaseolus vulgaris* L.) varieties, Kentucky Wonder and Kentucky Wonder Wax, were used in the present study.

Procedure.—A detached-leaf method somewhat similar to one previously reported (8) was used. Plants

[1] Accepted for publication November 26, 1957.

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with uncrinkled partially expanded primary leaves (about 1½–2 in. wide) and with trifoliate leaves tightly folded within the terminal bud were selected. Primary leaves were selected immediately following the crinkled stage and were detached by severing the petiole close to the leaf blade.

The detached leaves were inoculated with a ¼-in. hog's-hair brush (17) saturated with inoculum, which consisted of juice expressed from the leaves of systemically invaded Black Valentine bean plants and then diluted 1:40,000 with a 0.1 M phosphate buffer (pH 7). Sufficient 320-mesh Carborundum was added to make a concentration of 1 per cent. (This inoculum was dilute enough so that it produced well-isolated local lesions on leaves not exposed to high temperature.)

The detached leaves were placed on tissue paper, which rested on a cloth towel. A fresh area of the paper was used for each inoculation to prevent contamination.

The leaves were incubated in Petri dishes on 1 per cent Bacto agar, with no nutrients added. One group of leaves was kept at 21±2°C in the dark for different periods (24, 48, or 72 hours). A comparable group was maintained in the dark at 32±1°C for each of the same periods. The temperatures 21° and 32° are hereafter designated as low and high temperature, respectively. Incubation in the dark either preceded or followed inoculation; when it followed inoculation, it started after a delay of 0, 24, 48, or 72 hours. After incubation for one of these periods, all leaves were then kept at the lower temperature (21°C) but were illuminated 12 hours daily (700 foot-candles supplied by fluorescent tubes) for the remainder of an experiment. An experiment lasted 6 days following inoculation.

Virus assay.—Samples composed of an equal number of uniform disks (6-mm diameter) were collected from each leaf, frozen, and stored. Each disk included either 1 lesion or a necrotic vein area.

The samples were ground individually in a mortar with a pestle. They were then diluted with appropriate volumes of the phosphate buffer (with Carborundum) previously described. Highly concentrated virus samples were diluted sufficiently so that lesion counts could be considered approximately proportional to the concentration of infectious virus in the extract (14).

A single sample from a detached leaf exposed to a high-temperature period was used to inoculate 1 primary leaf of each of 3 intact Pinto bean plants, and the opposite leaves were inoculated with the standard inoculum (1:40,000 dilution of juice) previously described. Samples from leaves exposed to the same period of low temperature were similarly compared with the standard inoculum. From the ratios obtained, estimates were made of the differences in the relative concentration of virus in the samples obtained from leaves given different treatments.

EXPERIMENTAL RESULTS.—*Effect of high temperature immediately after inoculation on necrosis distribution.*—*1) Length of high-temperature exposure.*—High temperature in the dark induced vein necrosis on primary leaves infected with SBMV (Fig. 1). As the length of exposure to high temperature increased, vein necrosis increased and isolated local lesions decreased. Frequently, with 72 hours of high temperature immediately after inoculation, necrosis was exclusively associated with veins and veinlets (Fig. 1, D, F).

2) *Time after inoculation of first exposure of leaves to high temperature.*—A delay of 24, 48, or 72 hours in starting the high-temperature exposure changed the relative proportions of vein necrosis and local lesions. The longer the high-temperature treatment was delayed the less frequently necrosis was associated with veins and the more frequently necrosis was associated with local lesions. This variation in the distribution of necrosis was similar for the 3 periods studied (24, 48, and 72 hours). When the high-temperature period began 72 hours after inoculation, the restriction of necrosis to local lesion areas was essentially the same as when no high-temperature treatment was given.

Effect of high temperature prior to inoculation.—A 24-hour high-temperature period prior to inoculation favored local-lesion formation, but occasionally it induced necrosis of a few veins.

On such leaves, the lesions were larger, on the average, than those on leaves not so treated. The number of local lesions that occurred in 5 experiments ranged from 2 to 6 times as great in the leaves exposed to high temperatures as in leaves kept at low temperature before inoculation. Increase in lesion number and size with high temperature pretreatment has been observed for other virus-host combinations (7, 21, 22).

A 48- or 72-hour high-temperature period also favored local-lesion formation, but these periods of high temperature usually resulted in slightly more necrosis of veins than occurred with low temperature. The frequency and extent of this necrosis were usually less, and never greater, than occurred with only 24 hours of high temperature immediately after inoculation.

Relation between necrotic areas and location of virus.—The assay procedure previously described was used to locate areas in the leaves where the virus was present in the greatest amount. The sampling procedure was modified in the following way. A necrotic region was removed with a cork borer (6-mm diameter), and the surrounding non-necrotic region was cut out with another cork borer (9-mm diameter) in such a way that the 6-mm region was within the 9-mm region. The necrotic region was either a local lesion or associated with necrotic veins. Paired necrotic and non-necrotic samples were compared (with correction for the difference in sample area) by the local-lesion assay procedure described. A much larger amount of virus was present in necrotic regions than in the adjacent non-necrotic areas (Table 1). In some experiments, no virus could be detected in the symptomless tissue. This suggested that distribution of necrotic symptoms reflected to some degree the distribution of virus.

Comparison of the amounts of infectious SBMV isolated from local lesions and from necrotic-vein areas.—The results of several experiments (Table 2) indicate that necrotic areas from leaves held at high

TABLE 1.—*Relative infectivities of extracts from local lesions, from necrotic vein areas, and from non-necrotic areas surrounding the necrosis on bean leaves inoculated with southern bean mosaic virus and given different temperature treatments*

Kind of sample	Ratio of number of lesions induced by indicated sample to number induced by standard inoculum [a]		Ratio (necrotic: non-necrotic)
	Necrotic	Non-necrotic	
Local lesion	11:1	0.0:1	11:0
Necrotic vein	161:1	0.2:1	805:1

[a] Each number is based on the average obtained from the assay of 6 samples by the inoculation of 3 plants per sample. Average number of lesions ranged from 0 to 500.

TABLE 2.—*Ratios of the number of local lesions induced by extracts from uniform disks from leaves inoculated with southern bean mosaic virus and held at high temperature (32°C) to the number of lesions induced by similar extracts from disks of inoculated leaves held at low temperature (21°C)*

Period at high temperature (hours)	Lesion ratio [a]			
	Test 1	Test 2	Test 3	Average
0[b]	1.1:1	1.1:1		1.1:1
24	14:1	5:1	8:1[c]	9:1
48	32:1	60:1	72:1	55:1
72	60:1	112:1	117:1	96:1

[a] Average number of lesions ranged from 10 to 500 per leaf.

[b] Held at low temperature for the entire 6 days.

[c] All ratios were significant at the 1 per cent level except this ratio, which was significant at the 5 per cent level.

temperature contained more virus than did those from leaves kept low at temperature; the difference was statistically significant. Twenty-four hours was sufficient to cause a marked increase, and longer periods resulted in further increases. The data indicate that much more infectious virus was associated with the necrotic-vein samples from leaves exposed to high temperature than with the isolated local-lesions.

DISCUSSION.—Our results indicate that exposure of leaves to high temperature after inoculation facilitated invasion by SBMV of veinal cells rather than interveinal cells. It would appear that much of the effect of high temperature after inoculation was on a stage in the development of disease later than that of primary infection, since a 24- and 48-hour delay in the start of high temperature did not eliminate an increase in vein necrosis.

High temperature prior to inoculation also facilitated invasion by SBMV. The increase in invasion differed from that induced by treatment after inoculation in that more individual areas of necrosis occurred and each area remained largely interveinal rather than veinal.

One possible explanation for the difference in results between preinoculation and postinoculation high-temperature treatments is that some of the changes in leaf physiology caused by high temperature were only temporary and did not persist after virus inoculation. With postinoculation high-temperature treatments, however, these changes occurred in the presence of virus and thus had an immediate effect on the location of virus multiplication. It is important to note, though, that changing the temperature 72 hours after inoculation had no apparent effect on virus distribution, even though the change in temperature occurred during virus multiplication. The possibility that the effect is partly on the virus is not ruled out by our study.

Since the total necrotic area in leaves exposed to high temperature was greater than that in leaves exposed to low temperature, it would appear that the amount of virus in each leaf exposed to high temperature was proportionately greater than the amount in each leaf exposed to low temperature. The full magnitude of this difference, however, was not reflected in the sampling method used.

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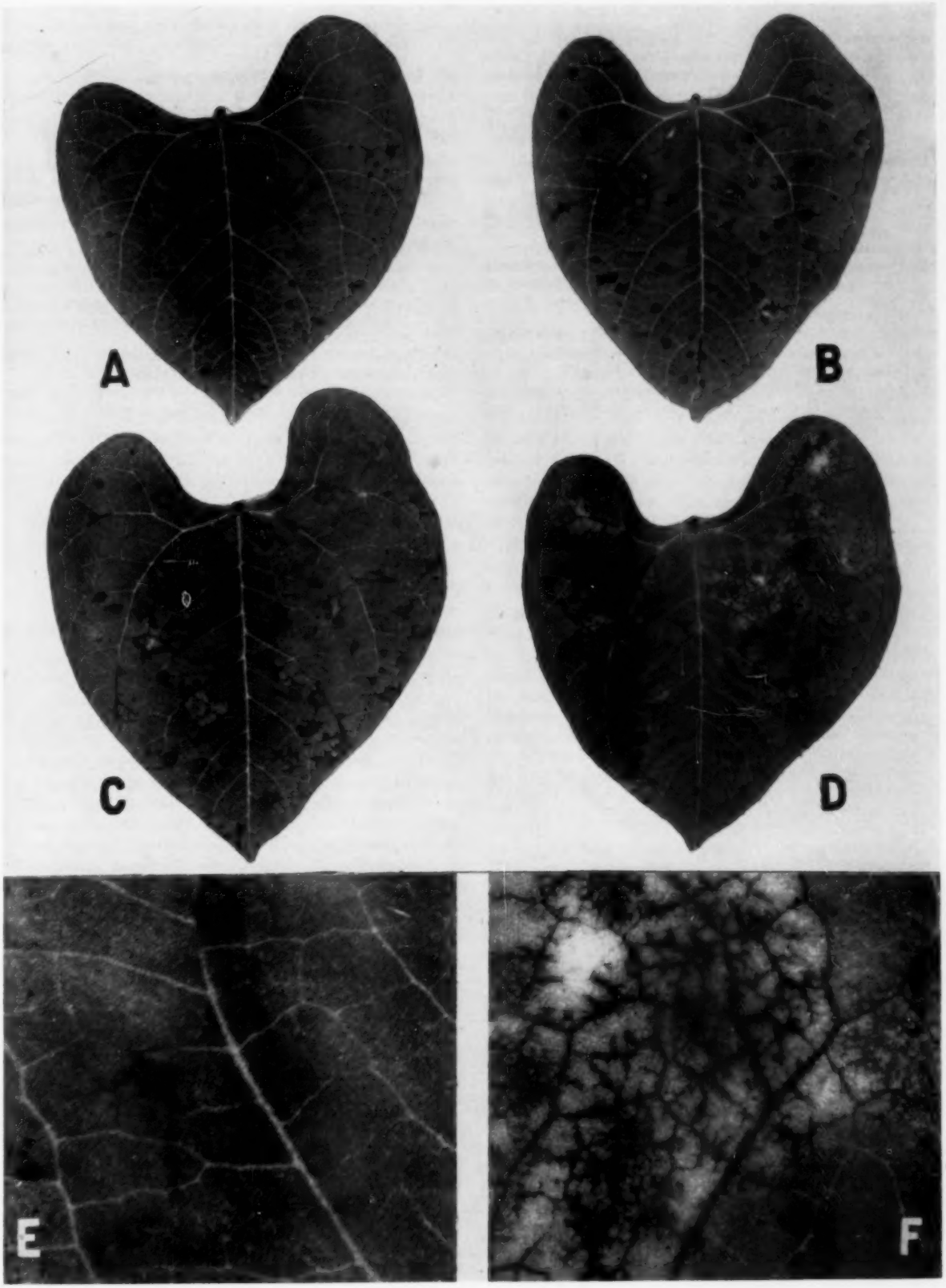
FIG. 1. Vein necrosis and local lesions in bean leaves inoculated with southern bean mosaic virus and exposed to high temperature for different periods. A) Not exposed to high temperature (control). B, E) 24-hour exposure. C) 48-hour exposure. D, F) 72-hour exposure.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THE RELATIONSHIPS OF SEVEN SPECIES OF ASCOCHYTA OCCURRING IN NORTH CAROLINA [1]

D. F. Crossan[2]

SUMMARY

Isolates conforming to the descriptions given for *Ascochyta phaseolorum*, *A. abelmoschi*, *A. gossypii*, *A. althaeina*, *A. nicotianae*, *A. capsici*, and *A. lycopersici* were obtained from naturally infected hosts in North Carolina. The spores of all isolates were similar morphologically. When the growth response to hydrogen-ion concentration was plotted for representative isolates from each host, the resultant curves were bimodal. The minimum between the 2 maxima occurred between pH 4.5 and pH 5.5. All isolates made slight growth at a hydrogen-ion concentration as high as pH 2.8 and as low as pH 7.4, with an optimum between pH 5.5 and pH 6.0. Isolates from a single host varied widely in cultural types, and these types were common to all isolates. The optimum temperature for growth of a majority of the isolates was 24°C, but the optimum for some was 20°C. All isolates grew at temperatures between 8° and 30°C. The optimum for growth of 1 isolate from snapbean was between 16° and 20°C. The species of *Ascochyta* associated with diseases of snapbean, cowpea, soybean, pepper, tomato, eggplant, tobacco, cotton, okra, and hollyhock in North Carolina were shown to be morphologically, physiologically, and pathogenically similar. In cross-inoculation studies, symptoms produced on each host were identical, irrespective of the original source of the inoculum. It is proposed that the species *A. abelmoschi*, *A. gossypii*, *A. althaeina*, *A. nicotianae*, *A. capsici*, and *A. lycopersici* be tentatively considered as synonomous with *A. phaseolorum*, which has priority as the earliest named species in Saccardo (1884). The Ascochyta leafspot diseases of hollyhock, pepper, tobacco, and eggplant are recorded for the first time in North Carolina.

Ellis (7) reported that isolates of *Ascochyta abelmoschi* Harter obtained from okra (*Hibiscus esculentus* L.) were pathogenic on snapbean (*Phaseolus vulgaris* L.) and Lima bean (*Phaseolus lunatus* L.), and that isolates of *A. phaseolorum* Sacc. obtained from the last 2 hosts were pathogenic on okra. On the basis of similarity of disease reaction and spore size, he suggested that perhaps only 1 fungus was responsible for both diseases. A review of the literature revealed that several species of *Ascochyta* have been reported as pathogens of malvaceous plants (2, 6, 9, 13, 18, 19) and that *A. phaseolorum* had a wide host range within the *Leguminoseae* (4, 7, 10, 16, 20). It seemed probable that many of these species might be closely related

[1] Accepted for publication November 26, 1957.
Contribution from Plant Pathology, North Carolina Agricultural Experiment Station, Raleigh. Published with the approval of the Director of Research as Paper No. 828 of the Journal Series. A portion of a thesis presented to the graduate faculty of North Carolina State College in partial fulfillment of the requirements for the Doctor of Philosophy degree.

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or identical and that this relationship might also apply to species reported as pathogens of plants in other families as well (1, 3, 8, 11, 12, 13, 14, 15).

Consequently, comparative studies were made of isolates conforming to the descriptions of several described species of *Ascochyta*. These included *A. phaseolorum* from snapbean, soybean (*Glycine max* (L.) Merr.), and cowpea (*Vigna sinensis* (Torner) Sevi); *A. abelmoschi* from okra; *A. gossypii* Syd. from cotton (*Gossypium hirsutum* L.); *A. althaeina* Sacc. et Bizz. from hollyhock (*Althaea rosea* (L.) Cav.); *A. nicotianae* Pass. from tobacco (*Nicotiana tabacum* L.); *A. lycopersici* Brun. from tomato (*Lycopersicon esculentum* Mill.) and eggplant (*Solanum melongena* L.); and *A. capsici* Bond.-Mont. from pepper (*Capsicum annuum* L.). The Ascochyta leafspot diseases of hollyhock, pepper, tobacco, and eggplant were recorded for the first time in North Carolina. Reciprocal pathogenicity studies were made with isolates of the previously listed species of *Ascochyta* under controlled conditions and with certain isolates in field plots. Cultural and morphological comparisons of the various species were also made. A preliminary report has been published (5).

MATERIALS AND METHODS.—More than 200 isolates derived from single spores of species of *Ascochyta* were obtained during the 1952 and 1953 growing seasons from plants of cotton, okra, hollyhock, snapbean, soybean, cowpea, tobacco, pepper, tomato, and eggplant. The isolates were maintained on potato-dextrose-agar slants at approximately 5°C. Although a few isolates produced pycnidia in potato-dextrose-agar cultures at 25°C, the majority did not. Since all isolates formed sporulating pycnidia when grown on sterilized potato cylinders at 25°C, this medium was used for the production of inoculum. Inoculum was prepared by grinding the potato cylinder in distilled water in a Waring Blendor and straining the suspension through cheesecloth. The inoculum contained approximately 10,000 spores per ml. The following were used in the inoculation studies as test plants: Black Beauty eggplant; Marglobe tomato; 402 tobacco; California Wonder pepper; Louisiana Green Velvet okra; Coker's 100 W cotton; Roanoke soybean; Tendergreen, Kentucky Wonder, and McCaslan snapbean; Henderson Bush and King of the Garden Lima bean; Sugar Crowder and Black Eye cowpea; Kobe lespedeza (*Lespedeza striata* (Thunb.) H. et A.); Oklahoma alfalfa (*Medicago sativa* L.); Spanish peanut (*Arachis hypogaea* L.); White Sweet Dutch clover (*Trifolium repens* L.) Alaska pea (*Pisum sativum* L.), and Jean Elizabeth chrysanthemum (*Chrysanthemum marifolium* (Ramat.) Hemsl.).

All plants were grown in steamed soil in 4-in. clay pots. Replicated pots of plants in different stages of growth were inoculated by spraying them with spore suspensions; the inoculated plants were held for a 48-hour period in a moist chamber at approximately 100 per cent relative humidity and at a temperature of 65°±3°F. They were then placed on benches under fluorescent lights and held at 65° for 7–12 days, when results were recorded. The inoculation results were evaluated by means of the following relative disease severity scale: 1) slight, less than 25 per cent of aboveground plant parts infected; 2) moderate, 26–50 per cent infected; 3) severe, 51–75 per cent infected; and 4) very severe, 76–100 per cent of aboveground plant parts infected or plants dead.

EXPERIMENTAL RESULTS. — *Pathogenicity studies under controlled conditions.*—An extensive series of inoculation experiments with several isolates from each of the above hosts, with 1 isolate each of *A. phaseolorum* and *A. lycopersici* obtained from Baarn, Holland (Centralbureau voor Schimellcultures), and with 1 isolate of *A. chrysanthemi* obtained from the American Type Culture Collection, were made between November, 1952, and March, 1954. In given inoculation experiments, pots of plants of each variety in different stages of growth were used. With cotton, okra, and snapbean, these included seedlings with 2–4 expanded leaves, plants with 5–7 expanded leaves, and plants bearing immature fruits. The remaining plants tested usually had 4–10 expanded leaves at the time of inoculation. The plants were rated for disease severity and pycnidial production. The same hosts were used with all isolates studied.

Essentially similar inoculation results were obtained with the North Carolina isolates obtained from okra, hollyhock, cotton, bean, cowpea, soybean, tomato, pepper, eggplant, and tobacco. Symptoms caused by a particular isolate on the leaves, stems, and fruits of the various test plants were usually identical to those produced with isolates from any of the other hosts (Fig. 1). Table 1 gives results that are representative of those obtained in inoculation tests. The isolates varied somewhat in virulence but with few exceptions were most virulent on okra, snapbean, and cotton and somewhat less severe on tomato, pepper, and eggplant. Cowpea plants were severely affected only by the isolates of *A. lycopersici* from tomato and of *A. capsici* from pepper. Soybeans were severely affected by the North Carolina isolate of *A. lycopersici* from tomato but only slightly affected by the eggplant isolate of this species. Tobacco, lespedeza, and hollyhock were not severely affected by any of the isolates tested. No disease lesions were observed on alfalfa and pea plants. Although there was some variation in the relative susceptibility or resistance of particular hosts to certain isolates, this variation was not associated with pathogenicity to the original host from which the culture was obtained. For example, isolates from cotton were uniformly more pathogenic to snapbean than to cotton. Isolates from soybean were very pathogenic on snapbeans and okra and only mildly pathogenic to soybeans. Similarily, isolates from hollyhock were only weakly pathogenic to hollyhock. *A. chrysanthemi* was parasitic only to chrysanthemum.

Inoculations made with the isolate of *A. phaseolorum* from Baarn, Holland, produced only a flecking reaction on the McCaslan bean variety and no reaction on any of the other common bean varieties, such as Tendergreen. A flecking reaction also resulted on okra and

on tomato. It produced definite lesions on the cotyledons of cotton and leaves of lespedeza. Two of 18 inoculated okra plants developed stem lesions that girdled the plants and eventually killed them. Pycnidia were produced on infected plants. This isolate also differed from the others in response to temperature and to hydrogen-ion concentration, as will be pointed out later. Furthermore, the results obtained here differed considerably from those reported by Sneep (16), who worked extensively with *A. phaseolorum* in Holland. Similarly, *A. lycopersici* obtained from Baarn did not react in a fashion comparable to the other isolates. This isolate was quite pathogenic to the solanaceous hosts but only slightly pathogenic to cotton. It caused a flecking reaction on okra and was nonpathogenic to all leguminous hosts inoculated except Lima bean and lespedeza. Symptoms were comparable to those produced by isolates of *Ascochyta* from tomato obtained in North Carolina.

Certain facts not evident in the table should be pointed out. Pycnidia were produced in 5–7 days after inoculation on the plant parts infected. All the isolates listed usually formed pycnidia on cotton, snapbean, okra, soybean, tobacco, pepper, tomato, eggplant, cowpea, and lespedeza. No pycnidia were produced on hollyhock, even when this host was inoculated with isolates originating from hollyhock. *A. althaeina* from hollyhock and *A. capsici* from pepper formed no pycnidia on tobacco, and *A. lycopersici* from tomato formed no pycnidia on lespedeza and tobacco. Similarily, *A. nicotianae* did not form pycnidia on lespedeza. The isolate of *A. lycopersici* from Holland formed pycnidia only on tomato, pepper, and eggplant. No pycnidia were formed on any of the crops tested with the isolate of *A. phaseolorum* from Holland. All isolates that formed pycnidia produced them on infected plant parts from the seedling stage of growth to maturity.

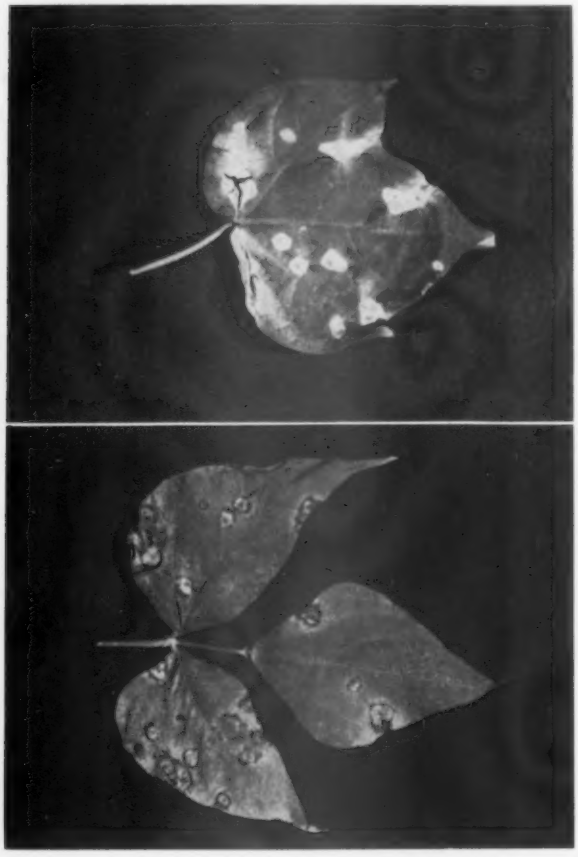

FIG. 1. Upper: Symptoms on cotton leaf following inoculation with an isolate of a species of *Ascochyta* from snapbean. Lower: Symptoms on snapbean following inoculation with an isolate of a species of *Ascochyta* from okra.

Pathogenicity studies in field plots.—An isolated plot at the McCullers Branch Experiment Station, McCullers, North Carolina, was seeded with Cokers 100-W cotton. The young plants were inoculated with a spore suspension of a culture of *Ascochyta* originally isolated from cotton. Over 70 per cent of the cotyledons of the plants developed typical symptoms of Ascochyta leaf spot. At this time, Tendergreen and Kentucky Wonder snapbean, Roanoke soybean, Louisiana Green Velvet okra, and Henderson Bush lima bean were planted between the rows of infected cotton. Plants of California Wonder pepper, Black Beauty eggplant, 402 tobacco, and double Pink hollyhock were transplanted to additional rows in the plot.

The plot was irrigated with overhead sprinklers 6 times within a 12-day period. Approximately 20 per cent of the cotton foliage and 10 per cent of the foliage of the bean and okra plants became infected with Ascochyta. Occasional Ascochyta leaf spots were observed on the other crops.

An experimental plot of okra and snapbeans growing at the Mountain Fruit and Vegetable Station, Fletcher, North Carolina, became severely diseased with Ascochyta leaf spot during the 1952 growing season. In 1953, this area was planted to the various crops under consideration in this study. The plot was divided into 2 sections, and plantings were made on 2 different dates. Disease readings were taken on the plantings at intervals during the growing season. In the first planting, over 40 per cent of the cotton and okra foliage developed typical Ascochyta leaf spot. Two plants of tobacco variety 402 and 5 plants of pepper variety California Wonder that were adjacent to severely infected okra plants developed Ascochyta leaf spots on the lower leaves. Approximately 1 per cent of the lower foliage of the soybean, cowpea, and snapbean varieties developed lesions typical of Ascochyta in the second planting, except that Ascochyta lesions formed on the lower leaves of 2 Black Beauty eggplant plants and the lower leaves of 3 Marglobe tomato plants became diseased. Although conditions were not particularly favorable for disease development, Ascochyta leaf spots were observed on leguminous, malvaceous, and solanaceous plants growing in the plots. Cotton and tobacco had not been grown previously on this land, yet the early development of the disease on cotton was severe and a number of lower leaves of tobacco became infected.

Colony morphology.—Since the isolates from North Carolina used in the present study were thought to represent a common population, comparisons of the colony morphology of 200 single-spore isolates in Difco potato-dextrose agar were made. The cultures were placed into 7 categories.

Type I had submerged mycelium that was at first hyaline but became dark olivaceous in the center and lighter toward the edge of the colony. The periphery was opaque white. Aerial mycelium that formed after 7 days appeared as intermingled tufts of gray and white mycelium and covered the plate in approximately 11 days.

Type I-A was similar to Type I except that growth rate was about one-half as rapid.

Type II had submerged mycelium that was at first hyaline, then became dark in the center. Aerial mycelium by 11 days was a uniform tufted gray and covered the plate.

Type II-A was the same as Type II, except that growth rate was about one-half as rapid.

Type III made early growth as in Types I and II, but aerial mycelium became mealy in appearance, not tufted, and was gray in color.

Type IV had submerged mycelium as in Types I and II, but aerial mycelium was predominantly white and became cream or tan with age.

Type V had submerged mycelium as in Types I and II, but by 11 days very little aerial growth was produced. Pycnidia were produced in a crusteose mass over the agar surface.

The majority of isolates fell into Types I and II; however, a few completely white isolates from different hosts were found and occasional cultures that were predominantly pycnidia producers were isolated. The wide range of variation that existed within isolates from the same host is illustrated in Figures 2 and 3. The predominant tufted gray or gray and white type is represented in isolates from both hosts. Cultural types varied among isolates from different sources, but this variation was no greater than that among isolates from the same source.

Morphology.—Attempts to obtain original type specimens from Europe were unsuccessful. Herbarium material of original collections of *A. abelmoschi* and *A. boltshauseri* obtained from the Mycological Collections, U. S. Department of Agriculture, and specimens of *A. gossypii* collected in North Carolina in 1924, were examined. In addition to the above, examinations were made of all of the field specimens collected in North Carolina during 1952 and 1953. The various *Ascochyta* species examined possessed a similar spore morphology and a similar range in spore size. Since the spore size and morphology of the various species exhibited a certain amount of variation, measurements and drawings were made to determine whether any of the species were sufficiently different in these respects to be separated from the others. The pycnidia of

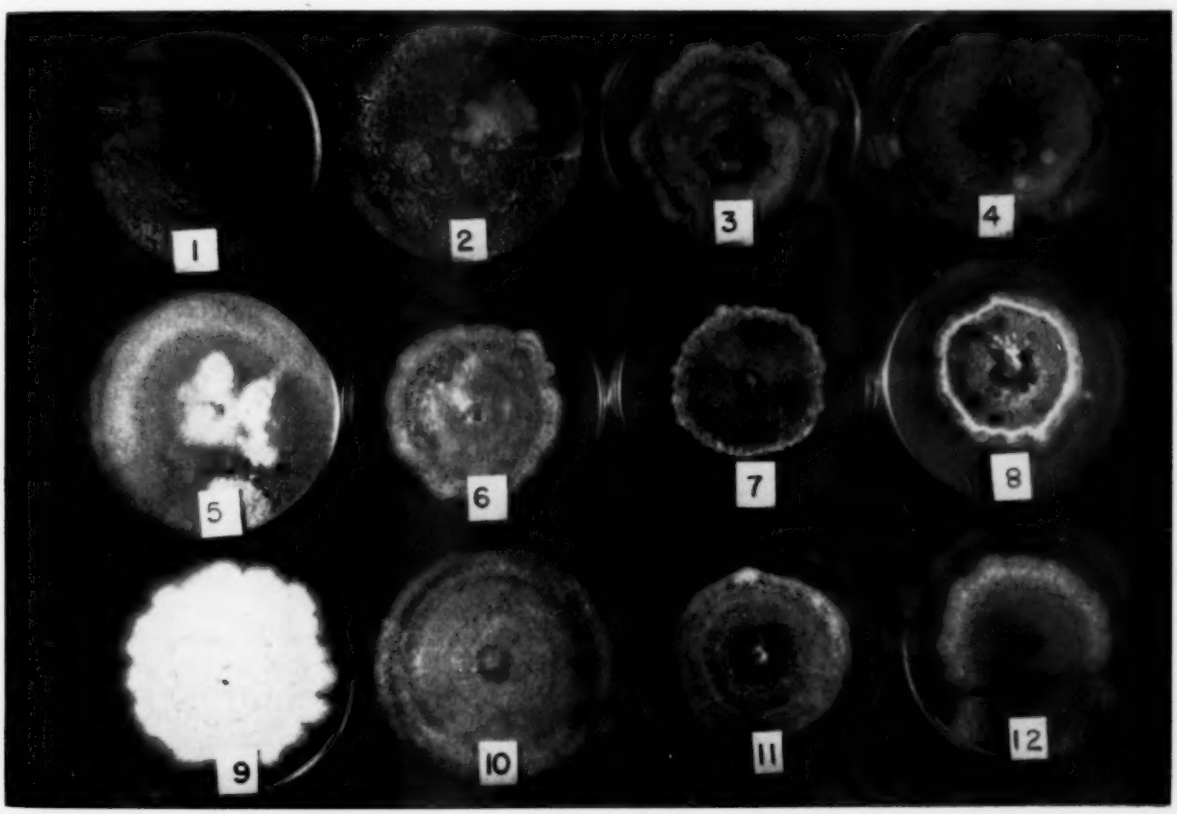

FIG. 2. Variation in cultural characteristics of 12 single-spore isolates of a species of *Ascochyta* from cotton.

all the species were typically epiphyllous, and at first were light brown in color but turned dark with age.

To compare the sizes of spores, a series of measurements was made of spores from various sources. Fifty 1-celled and 50 2-celled spores were measured from each naturally infected host. A similar number of measurements were made of spores produced in culture and on experimentally inoculated hosts. A total of 5900 spores from 49 different sources were measured. The data were analyzed statistically and LSD values obtained for comparisons of the means of any 2 sets of measurements. Statistically significant differences sometimes occurred between different populations of spores originating from the same source. Spores from isolates from the same source and grown on different substrates showed significant differences, as did spores produced on different hosts following inoculation with spores from the same single-spore culture. The data indicated that the variation of the means of these populations was as great among isolates from the same source as it was among isolates from different sources.

Camera lucida drawings were made from water mounts of spores originating from different sources (Fig. 4). The drawings were made of spores produced at the same time and under the same environmental conditions. The 3 populations appeared to be identical. The same may be said of any of the other comparisons of camera lucida drawings of spores produced on potato cylinders. The spores of all isolates that produced pycnidia on potato-dextrose agar following flooding of plates with spore suspensions were slightly larger than those produced on potato cylinders. It should be noted also that the spores from a given source varied with respect to constriction or lack of constriction at the septum, the position of the septum, and the number of septa. Two-septate spores both from naturally infected hosts and from culture were observed occasionally in all cultures and on artificially inoculated hosts.

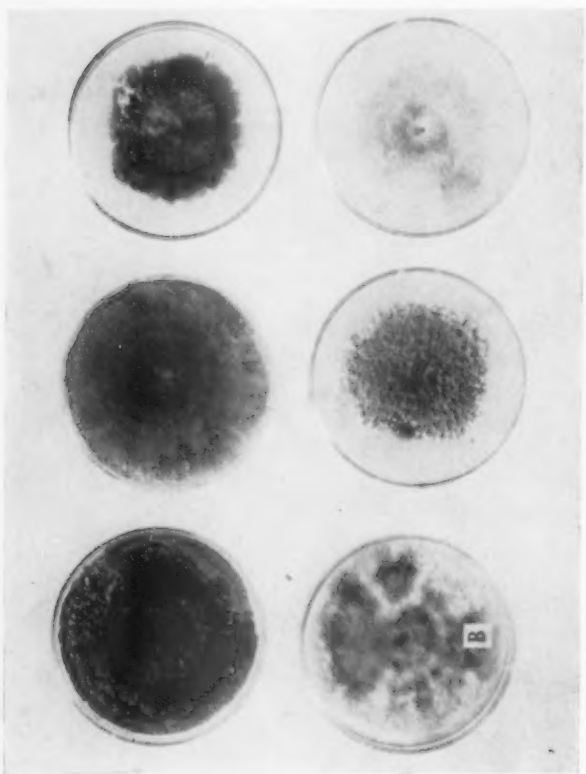

FIG. 3. Colonies of 6 single-spore isolates of a species of *Ascochyta* from snapbean.

Pycnidial production.—Pycnidia were produced on sterilized potato cylinders within 3–4 weeks. Pycnidiospores from exuded spore masses were transferred to different media to determine whether predominantly pycnidia-producing cultures would result. The growth was essentially vegetative on potato-dextrose agar, although a clump of pycnidia often formed at the point where the cultures were seeded. On potato-sucrose agar, many isolates produced a luxuriant vegetative growth followed by the production of pycnidia at the center of the colony. The aerial growth gradually became suppressed, and the area of pycnidial production continued to extend outward during several weeks' development, forming a crust of pycnidia several centimeters in diameter.

An additional method was used to induce sporulation. Heavy suspensions of spores were prepared by washing the pycnidial ooze from potato cylinders. Approximately 1 ml of this suspension was pipetted aseptically onto the potato-sucrose agar medium. When a sufficiently dense suspension of spores was used, the area of the plate that was flooded rapidly developed a crust of pycnidia. Vegetative growth would occur outward from the edge of the flooded area. This method was used to produce spores for tests on the effect of hydrogen-ion concentration on growth in culture.

Temperature relationships.—The isolates from North Carolina have been shown to have identical host ranges and similar ranges in spore size and morphology and in colony morphology. Tests were undertaken to determine whether they would also have similar response to temperature. The majority of isolates from all hosts had an optimum of 24° C. Growth occurred at 8° and 28° but not at 30° after 6 days. Very slight growth occurred after 18 days at 30°. A number of isolates from different hosts grew as well at 20° as at 24°. One isolate from bean grew best between 16° and 20° and 1 isolate from okra grew best at 28° but did not grow at 30°. The isolates of *A. phaseolorum* obtained from Baarn, Holland, made optimum growth between 20° and 24° but did not grow at 28°. *A. lycopersici* from this source behaved similarly, except that a trace of growth occurred at 28°.

The effect of temperature on spore germination was also determined for several isolates from different hosts. Spore suspensions were aseptically pipetted onto potato-dextrose agar plates and the plates kept at varying temperatures. Measurements of the length of germ tubes 12–15 hours after seeding showed that the rate of germ tube development was influenced by temperature in the same manner as was colony growth,

with 20°–24° as the optimum for germination and development of most of the isolates. The results indicated that isolates of Ascochyta from the same host may fall into 2 groups with respect to response to temperature—those that grow equally well at 20° and 24° and those that grow best at 24°.

Hydrogen-ion concentration.—To determine whether the cultures differed in response to hydrogen-ion concentration, 18 of the isolates from North Carolina, isolates of *A. boltshauseri*, *A. phaseolorum*, and *A. lycopersici* from Baarn, Holland, and an isolate of *A. imperfecta* from Wisconsin were studied in liquid culture.

A preliminary screening of media and buffers indicated that potato-dextrose broth and a buffer mixture prepared from glycine (1.9 g), citric acid (1.3 g), potassium dihydrogen phosphate (1.9 g), and distilled water (50 ml) were very satisfactory. Thirty-five ml of the broth was placed in each of the required number of flasks, which were then plugged and sterilized. By means of a sterilized pipette, 2 ml of the sterilized buffer mixture was added to each flask. The reaction of the resulting mixture, which averaged pH 4.0, was adjusted as required by aseptic addition of appropriate amounts of 1 N HCL or NaOH. Each series was replicated at least twice.

Two methods of seeding the broth mixtures were used. Where pycnidia were produced readily on potato-dextrose agar, the exuded spores were washed off and 1 ml of the resulting suspension was aseptically pipetted into each flask of buffered potato-dextrose broth. Two adjusted but non-seeded flasks at each hydrogen-ion level were kept as checks. With cultures that did not readily produce pycnidia, the isolates were grown on potato-dextrose agar plates and each flask was seeded with an 8-mm disk cut with a cork borer from the periphery of the colony. When spores were used as inoculum, the mycelium was harvested in 5 days; when cork-borer slices were used, mycelia were harvested after 8 days. The flasks were shaken several times each day during the test period. The mycelia were harvested by filtering on previously weighed filter papers. The mats were allowed to drain and to dry partially for a 24-hour period. They were then placed in a desiccating chamber under vacuum for an additional 24 hours, after which they were removed and weighed. The pH of the filtrate and reserve portions was determined at the time of filtering.

The growth-response curves of all isolates of suspected similarity were bimodal; those of *A. boltshauseri*

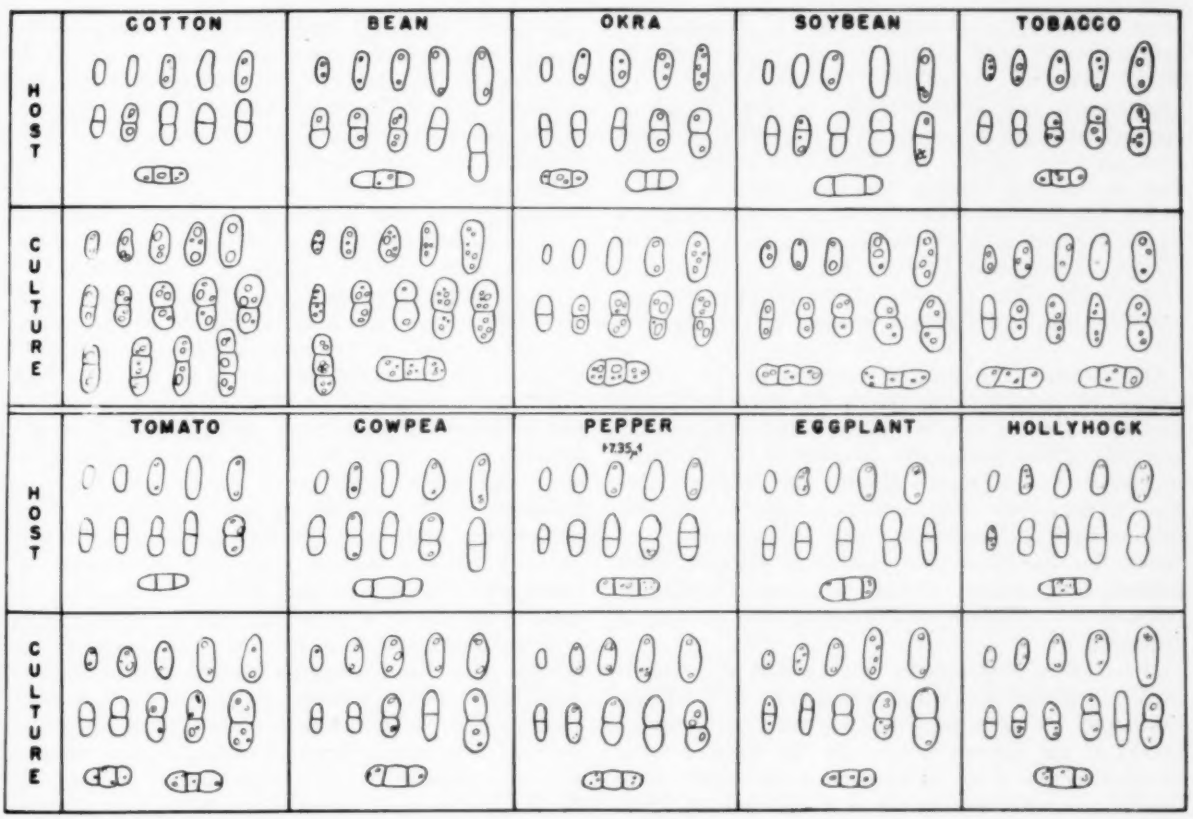

FIG. 4. Variation in size and shape of spores of a species of *Ascochyta* from 10 hosts and of spores from isolates from these hosts when such isolates were grown on artificial media.

TABLE 1.—*Pathogenicity of 11 isolates of Ascochyta species on 10 hosts*

Ascochyta species and source of isolate	Disease index [a] on leaves of indicated crop plants										
	Cotton	Snap-bean	Okra	Soy-bean	Tobacco	Pepper	Tomato	Egg-plant	Cowpea	Lespe-deza	Holly-hock
A. gossypii											
Cotton	2	3	3	2	1	2	2	2	1	1	1
A. phaseolorum											
Snapbean	3	3	3	2	1	1	1	2	1	1	1
Cowpea	2	2	2	1	1	2	2	2	2	1	1
Soybean	2	2	2	2	1	1	2	1	1	1	1
A. abelmoschi											
Okra	2	2	2	1	1	1	2	2	1	1	1
A. althaeina											
Hollyhock	2	2	2	1	1	1	1	2	1	1	1
A. lycopersici											
Tomato	2	2	4	1	1	1	1	2	3	1	1
Eggplant	2	2	4	1	1	1	1	2	2	_	1
Baarn [b]	1	0	Fleck	0	1	2	2	3	0	1	0
A. nicotianae											
Tobacco	3	2	3	1	1	1	1	2	_	1	0
A. capsici											
Pepper	2	2	4	3	1	2	2	2	4	1	1

[a] Rated on a relative scale of infection from 0 to 4 as follows: 0—no infection; 1—slight; 2—moderate, 3—severe, and 4—very severe infection of aboveground parts or death.

[b] Obtained from Centralbureau voor Schimellcultures, Baarn, Holland.

and *A. imperfecta* were not. The growth-response curves of *A. lycopersici* obtained from Holland also was bimodal; the point of the minimum (pH 6.2), however, was much higher than that of any of the other isolates. *A. boltshauseri* and *A. phaseolorum*, both obtained from Baarn, Holland, responded differently to extremes of pH. The pH range of *A. phaseolorum* and *A. imperfecta* was from pH 3.3 to approximately pH 6.0, and that of *A. boltshauseri* was from pH 4.6 to pH 6.5. All of the other isolates tested grew at reactions ranging from approximately pH 2.8 to pH 7.4. With the exception of *A. lycopersici* from Baarn, the minimum of the bimodal curves from the various isolates fell between pH 4.5 and pH 5.5 (filtrate pH). The majority of the isolates gave the greatest yield of dry matter between pH 5.0 and pH 6.0.

DISCUSSION.—The isolates representing the various species of *Ascochyta* in North Carolina were very similar and could not be differentiated either pathogenically or morphologically. In contrast, the European isolates used in these tests differed markedly in pathogenicity from these isolates. Before a definite relationship or lack of relationship can be established between the European and American isolates, more isolates from Europe should be tested. Herbarium material from Europe and America also should be compared.

It has been increasingly evident that the criteria used to separate the genera and species of the Fungi Imperfecti are often invalid. Early workers separated genera of the *Sphaeropsidales* on the basis of spore morphology and color. In the section *Hyalodidymae* of the *Sphaeropsidaceae*, the use of spore septation, spore color, and pycnidial morphology led to the erection of a number of genera such as *Ascochytula*, *Ascochytella*, *Diplodina*, *Stagonosporopsis*, and *Apiocarpella*. That these distinctions are invalid is evident by the fact that these genera are now considered synonymous with *Ascochyta* (17).

The use of the host plant as a means of distinguishing species with otherwise indistinguishable morphological features also has received considerable attention and has been used repeatedly as a basis for naming species. In the genus *Ascochyta*, there are valid species with restricted host ranges and also species that are known to have rather wide ranges of parasitism. In the latter group, there is great likelihood that numerous species have been differentiated on the basis of host alone when in reality only 1 species is involved. Furthermore, it is probable that more than 1 species of a given genus commonly attacks some or all of a given number of hosts. In order to distinguish among such species, it is necessary to determine the variation that exists in representative populations of the organisms and to determine whether or not sharply distinct differences occur among them. For species with distinct spore differences, such as *A. phaseolorum* and *A. boltshauseri*, the problem is not difficult. With *A. phaseolorum* and the 6 other species discussed in this paper, no such distinction occurs. To distinguish between such populations, one should know to what extent the variability found among cultures is continuous or discontinuous. In the present case, all representative isolates from North Carolina showed similar degrees of variation and conformity in all of the characters studied. The species *Ascochyta* associated with diseases of snapbean, cowpea, soybean, pepper, tomato, eggplant, tobacco, cotton, okra, and hollyhock in North Carolina were shown to be morphologically and physiologically very similar. No sharp distinctions could be drawn between any of the series of isolates

from different host plants. Identical symptoms were produced on each host irrespective of the original source of inoculum. These facts are believed to be sufficient grounds for considering the 7 species studied as representing 1 population with a wide range of parasitism. It is proposed that *A. abelmoschi*, *A. gossypii*, *A. althaeina*, *A. nicotianae*, *A. capsici*, and *A. lycopersici* be tentatively considered as synonymous with *A. phaseolorum*, which has priority as the earliest named species (1884) in Saccardo (13), pending further studies of additional herbarium material and isolates of this fungus from Europe.

PLANT PATHOLOGY
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ANTHRACNOSE OF AZALEAS[1]

P. D. Stathis and A. G. Plakidas[2]

SUMMARY

An apparently new leaf-spotting and defoliating disease of azaleas (hybrids of *Rhododendron* spp.) is described and named "azalea anthracnose." The disease, which affects both the Indica and Kurume groups of azaleas, occurred in severe epidemics every summer since 1954 in several nurseries in southeast Louisiana. The cause of the disease was found to be a species of *Colletotrichum* typical of the conidial stage of *Glomerella cingulata* (Ston.) Spauld. & Schrenk. Disease symptoms identical with those occurring naturally were reproduced on both Indica and Kurume azaleas by inoculation with pure cultures of this fungus. The fungus does not sporulate on the affected leaves as long as they remain alive and attached to the plant, but conidia form abundantly on the fallen dead leaves. Overwintered fallen leaves serve as the source of conidial inoculum the following spring and summer. No ascigerous stage was found either in culture or on the host. Both copper and organic fungicides were found effective in controlling the disease.

In the summer of 1954, a sudden outbreak of a serious leaf-spotting and defoliating disease of azaleas (hybrids of *Rhododendron* spp.) occurred in several nurseries in southeast Louisiana; the disease reappeared in the summers of 1955, 1956, and 1957. Several nurserymen in this area had thousands of badly defoliated, unmarketable plants during the 1954-55 season, thus suffering rather heavy losses. The disease appeared to be new and undescribed. No reference to such a disease was found in the literature, and neither the local nurserymen nor the pathologists in this laboratory who have been studying diseases of ornamental plants during the past several years had encountered it before. A study was undertaken to determine its cause and to test possible control measures. The disease is designated as "anthracnose," since its causal agent was found to be a species of *Colletotrichum*. This paper embodies the findings of this study. Brief preliminary reports have been published.[3, 4]

DESCRIPTION.—The disease is characterized by numerous small spots on both leaf surfaces. They are mostly circular in shape and range from 0.5 to 3.0 mm in diameter. These spots have definite margins and are olive brown to rusty brown in color (Fig. 1, A, B). With light infection, the lesions are discrete; in cases of heavy infection, the lesions coalesce and form large irregular spots. Young lesions have a smooth surface; older lesions are somewhat swollen and rough and are dark brown in color. On living leaves, the spots are superficial, are limited to the epidermal layer of cells, and have never been found to extend through the mesophyll to the opposite surface. As long as the leaves are alive and attached to the plant, the mycelium is intracellular and limited to the epidermal cells. Ingress is by direct penetration of the epidermis by means of a very slender peg that grows from the base of an appressorium on the surface. After the leaves fall, the mycelium grows intercellularly in the mesophyll tissues. The lesions occur for the most part on the leaves and only occasionally on tender young shoots. No fruiting bodies have been found on the lesions while the leaves are alive; however, conidia were produced abundantly in acervuli arising from the lesions on spotted leaves that were detached, allowed to dry for a day or so, and placed in a moist chamber. Abundant sporulation occurred also on naturally abscissed dry spotted leaves when these were kept moist for 24–48 hours. The fallen infected leaves serve as the source of inoculum. The first symptoms of the disease appear in late May or early June and always on young leaves. Mature leaves, in general, are free of spots. Heavily affected leaves fall prematurely, resulting in more or less severe defoliation. In extreme cases, defoliated shoots fail to produce new growth the next growing season (Fig. 1, C). Disease development and symptom expression are in general the same on both the Indica and Kurume azaleas.

ISOLATIONS.—Several hundred isolations were made from tissue platings of spotted leaves of several varieties of both Indica and Kurume azaleas collected in 4 localities, Folsom, Loranger, Baton Rouge, and New Orleans. Leaves bearing young spots were washed thoroughly with a brush under running water, immersed for 3 minutes in a disinfectant (1:1000 mercuric chloride, 50 per cent ethanol, or a saturated solution of calcium hypochlorite), and dried between layers of sterile filter paper. The leaves immersed in mercuric chloride were rinsed in 2 changes of sterile water before pressing between filter paper; those treated with calcium hypochlorite were not rinsed. Small pieces of tissue from the surface-disinfected leaves, cut from the margins of the lesions, were planted on potato-dextrose agar in Petri plates. The plates were incubated at room temperature for about 4 days, after which transfers were made from the margins of developing colonies to agar slants.

Between 70 and 80 per cent of the colonies develop-

[1] Accepted for publication December 9, 1957.

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[3] Stathis, P. D., and A. G. Plakidas, 1956. Anthracnose of azalea. Louisiana Agr. Expt. Sta. Ann. Rept. for 1954-55. p. 156.

[4] Stathis, P. D., and A. G. Plakidas, 1957. Anthracnose of azalea. (Abstr.) Phytopathology 47: 314.

ing from the leaf tissue yielded pure cultures of the same fungus, a species of *Colletotrichum* typical of the conidial stage of *Glomerella cingulata* (Ston.) Spauld. & Schrenk. This fungus was later grown on a variety of media (potato-dextrose, oatmeal, cornmeal, malt-extract, Czapek, and azalea-leaf-decoction agars and autoclaved azalea leaves), but in no case did it produce the ascigerous stage. Other fungi occasionally isolated were *Pestalotia* sp., *Alternaria* sp., *Fusarium* sp., and *Mycosphaerella* sp.

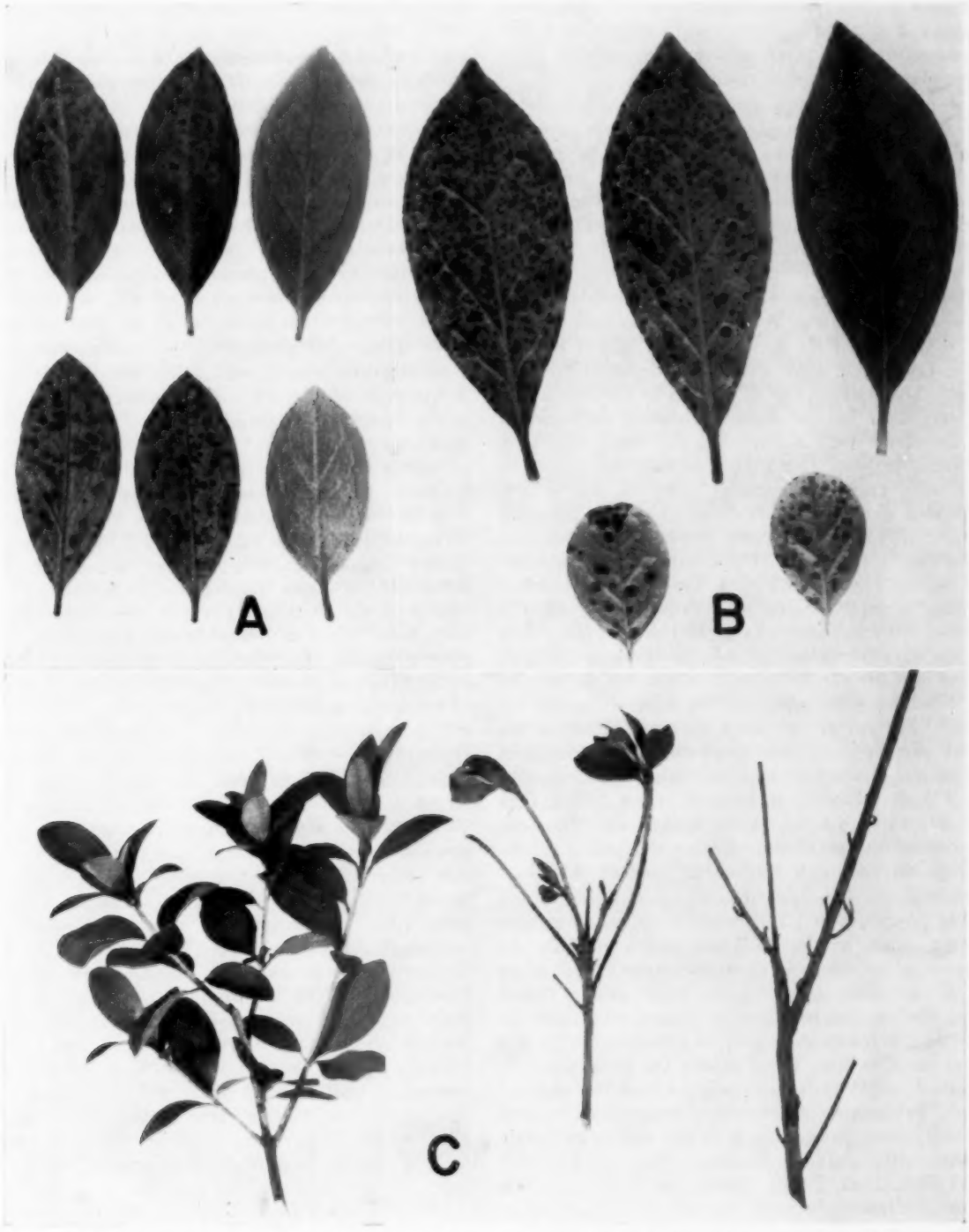

FIG. 1. Azalea anthracnose. A) Naturally infected leaves, with healthy ones shown for comparison. Top row, upper surface; bottom under surface. B) Leaves of Indica (top row) and Kurume (bottom row) azaleas artificially inoculated with pure-culture inoculum. C) Healthy (left) and 2 badly defoliated branches of azalea.

INOCULATIONS.—In a preliminary test, potted azaleas of 2 varieties, Formosa (Indica type) and Christmas Cheer (Kurume type), were inoculated separately by spraying with conidial suspensions from each of several tissue isolates of *Colletotrichum* sp. obtained from different varieties and different localities. Inoculations also were made with pure cultures of the other fungi (species of *Alternaria*, *Fusarium*, *Pestalotia*, and *Mycosphaerella*) that were occasionally isolated from tissue plantings of spotted azalea leaves.

Infection was obtained only on the plants inoculated with the *Colletotrichum* sp. Infection in every case was heavy, and the symptoms were typical of the disease as it occurred in nature. The fungus was reisolated from the artificially inoculated plants. None of the other fungi tested caused infection. The results of this preliminary experiment indicated definitely that the Colletotrichum was the cause of the disease.

Five varieties of Indica-type azaleas (President Clay, Pride of Mobile, Judge Solomon, Elegans, and Formosa), 6 varieties of the Kurume type (Salmon Beauty, Snow, Coral Bell, Pink Pearl, Hinodegeri, Christmas Cheer), and 9 monoconidial isolates of *Colletotrichum* sp. were used for the main inoculation experiments. All the plants were 2 years old and were potted individually in 10-in. clay pots. The Colletotrichum isolates were grown on oatmeal agar, on which they sporulated abundantly. The inoculum was prepared from 10-day-old cultures and consisted of a conidial suspension in tap water. The density of the inoculum was adjusted by means of a Levy-Hauser counting chamber to approximately 200,000 condia per ml, and 75 ml of this suspension was applied to each plant with a glass atomizer connected to a compressed air line. Plants of each variety were inoculated with each of the 9 isolates separately, and the experiment was repeated. Parallel checks were provided in which the plants were sprayed with water. Both the inoculated and control plants were kept in a large moist chamber for 3 days following inoculation, after which they were placed on a bench in the greenhouse. The temperature of the greenhouse during the period of the experiments fluctuated between 25° and 41° C.

Eight of the 9 isolates tested caused infection and typical symptoms in all 11 varieties. Lesions usually became visible by the third day and were fully developed by the tenth day after inoculation. One isolate proved completely nonpathogenic to all varieties tested. When the number and size of lesions were used as criteria, differences in degree of pathogenicity to different varieties were noted among the isolates tested. Isolates I and Ia (originally isolated from the varieties Judge Solomon and Formosa, respectively) were markedly more pathogenic than was any other isolate to the varieties Judge Solomon, Elegans, Formosa, Coral Bell, Pink Pearl, Hinodegeri, and Christmas Cheer and less pathogenic than was any other isolate to the varieties President Clay, Pride of Mobile, Salmon Beauty, and Snow. No significant differences in pathogenicity were apparent among the other 6 isolates. When the isolates were considered as a whole, differences in degree of susceptibility among the varieties tested were evident. Of the Indica varieties tested, Judge Solomon was the most susceptible. Of the 6 Kurume varieties tested, Hinodegeri was the most susceptible and Salmon Beauty, Snow, and Pink Pearl the most resistant. The lesions on the last 3 were very small.

In a third series of tests, the fungus used as inoculum was isolated from fallen overwintered leaves in the field. Acervuli bearing conidia were produced in profusion on these leaves after they were washed and placed in a moist chamber. Several monoconidial isolates were obtained, and these were used to inoculate plants comprising varieties of both Indica and Kurume azaleas. Infection resulting in typical symptoms of the disease (Fig. 1, B) was obtained in every case. The results of this experiment are considered of particular importance because, as already explained, the fallen overwintered leaves serve as the source of inoculum the following spring and early summer.

IDENTITY OF THE FUNGUS.—The only record found in literature of a species of *Colletotrichum* on azalea is the report of Ellis and Everhart[5] that contains a brief description of *C. azaleae* collected on *Azalea viscosa* in Florida. No fructifications were found on a fragment of the Ellis and Everhart specimen obtained from the National Fungus Collection, so no comparison was possible between *C. azaleae* Ell. & Ev. and the species of *Colletotrichum* that has been found to be the cause of azalea anthracnose in Louisiana. The lesions on the Ellis and Everhart specimen, however, were found to be entirely different from those of the present disease. Also, the sizes of the setae and conidia of the Ellis and Everhart fungus are smaller than those of the azalea anthracnose fungus.

The azalea anthracnose Colletotrichum appears typical of the conidial form of *Glomerella cingulata* (Ston.) Spauld. & Schrenk. Its fructifications on infected dead leaves were setose acervuli ranging from 73 to 140 μ in width. Setae, which were dark brown, straight to slightly curved, septate, and 5.4 × 92.2 μ in size, occurred abundantly in the acervuli on dead leaves but only rarely in culture. Conidia were hyaline, unicellular, and cylindrical, with ends rounded or somewhat pointed at 1 end. They were 5.1 × 16.6 μ in size and were borne on short, straight, hyaline condiophores. No ascospore stage was produced either in culture or on leaves. In culture, the Colletotrichum from azalea was found to be morphologically similar to a Colletotrichum isolated from crabapple fruit affected with typical bitter rot and also to the one causing dieback and canker of camellia, which Baxter and Plakidas[6] had determined to be a conidial form of *G. cingulata*. Inoculated into

[5] Ellis, J. B., and B. M. Everhart. 1895. New species of fungi. Bull. Torrey Botan. Club 22: 434-440.

[6] Baxter, L. W., and A. G. Plakidas. 1954. Dieback and canker of Camellias caused by Glomerella cingulata. Phytopathology **44**: 129-133.

apple fruit, the azalea isolate caused a rot similar in all respects to that caused by the bitter rot isolate. Conversely, inoculations of azaleas with the bitter rot fungus produced anthracnose lesions identical with those produced by the azalea Colletotrichum. In reciprocal inoculations of azaleas and camellias with Colletotrichum isolates from these 2 hosts, the azalea isolates caused cankers and dieback on camellia shoots whereas the camellia isolates were nonpathogenic to azaleas. It is concluded that the causal agent of azalea anthracnose is a conidial form of *G. cingulata*.

CONTROL.—Laboratory screening tests, in which the glass-slide spore-germination method was used, showed that very low concentrations of certain copper and organic fungicides were lethal to conidia of the causal fungus. The lowest concentrations of the 5 fungicides tested that resulted in complete inhibition of spore germination on glass slides were as follows: Bordeaux, 0.625 per cent; Tri-Basic Copper Sulphate (53% Cu as basic copper sulphate), 8 ppm; Cuprocide (90% Cu as cuprous oxide), 2 ppm; a 76 per cent formulation of zineb (zinc ethylenebis[dithiocarbamate]), 1 ppm; and a wettable powder, containing 50 per cent captan (*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide), 0.6 ppm. These findings indicated that the disease should be amenable to control by protective fungicidal sprays.

In greenhouse experiments, 5 fungicides, 4-4-50 Bordeaux and Tri-Basic Copper Sulphate, Cuprocide, zineb, (76% formulation), and captan at the rate of 3 lb. of formulated product per 100 gal. were tested under the following sets of conditions: 1) fungicides applied 24 hours after the plants were inoculated with a conidial suspension of the fungus; 2) fungicides applied 24 hours before inoculation; 3) fungicides and conidial suspension applied simultaneously (a double-strength suspension of the fungicide was mixed with an equal volume of the conidial suspension and the mixture sprayed on the plants); 4) the plants were sprayed with the protective fungicides, allowed to dry, then subjected to continuous artificial rain for various periods ranging from 24 to 144 hours, and then inoculated (the primary object of this experiment was to assay the relative tenacity of the respective fungicides).

Plants of Indica azalea Judge Solomon, a variety found to be very susceptible to the disease, were used in all the spray tests. All plants were kept in a moist chamber for 3 days following inoculation, after which they were placed on a greenhouse bench for 15 days for observation.

When the fungicide was applied 24 hours after inoculation, 24 hours before inoculation, and simultaneously with the inoculum, respectively, all 5 fungicides tested gave complete protection against infection (Table 1). When the sprayed plants were exposed to artificial rain for various periods of time before inoculation, the copper fungicides, particularly Bordeaux mixture and Cuprocide, proved more effective than did the organics, zineb and captan. Tri-Basic Copper Sulphate gave slightly longer protection than did zineb or captan, but it was definitely inferior to Bordeaux and Cuprocide. The adhesiveness and the resistance to washing by rain of Bordeaux and Cuprocide probably account for their superior performance.

No field or lath house spray tests were made by the writers; however, the experience of several nurserymen indicates that both copper and organic fungicides are effective in controlling this disease in the field or in lath houses. As soon as it became evident that the disease is induced by a fungus, and even before the identity of the causal fungus had been definitely determined, the nurserymen whose plants were affected were advised to spray; it was suggested that they try both copper and organic fungicides. Tri-Basic Copper-

TABLE 1.—*Relative amount of azalea anthracnose on plants of Indica azalea sprayed in the greenhouse with various protectants at various intervals prior to or after inoculation with Glomerella singulata* [a]

Time of inoculation and prior treatment	Relative amount of anthracnose [b] on plants sprayed with indicated fungicide					
	None	Bordeaux 4-4-50	Cuprocide [c]	Tri-Basic [c] Copper Sulfate	Captan [c]	Zineb [c]
24 Hours prior to spray	H	O	O	O	O	O
With spray	H	O	O	O	O	O
24 Hours after spray	H	O	O	O	O	O
After exposure of sprayed plants to rain for:						
24 Hours	H	O	O	T	T	O
48 Hours	H	O	O	T	T	T
72 Hours	H	T	T	L	L	L
96 Hours	H	T	T	L	H	H
120 Hours	H	T	L	M	H	H
144 Hours	H	L	H	H	H	H

[a] Plants sprayed with a conidial suspension, then held in moist chamber for 3 days.
[b] O = no disease, T = trace, L = light, M = moderate, H = heavy. Readings are based on 2 plants per treatment.
[c] Three lb. of formulated product per 100 gal. The captan and zineb formulations contained 50 and 76 per cent, respectively, of active ingredient.

Sulphate, C-O-C-S (56% Cu as basic copper chloride and basic copper sulphate), and ferbam were the fungicides most commonly used by the nurserymen, and all proved effective in checking the progress of the disease.

DISCUSSION.—No completely satisfactory explanation to account for the sudden appearance of this apparently new disease of azaleas can be offered at this time. It does not seem reasonable that the disease has been present for a long time and merely overlooked. Its extreme destructiveness argues against this. A case somewhat analogous is that of peach anthracnose, which appeared suddenly in epidemic form and caused severe losses to peach growers from 1946 to 1950 in the southeastern states.[7] In the case of peach anthracnose, the source of inoculum has been traced to profuse sporulation of the anthracnose fungus on blue lupines grown as a cover crop in peach orchards. In the case of azalea anthracnose, no recent introduction of a susceptible crop has been made and no alternate host of the fungus has been found.

G. cingulata is a common parasite of camellias, in which it causes bark cankers and dieback of shoots and branches. Camellias are often grown in close association with azaleas. The species of *Colletotrichum* (*Glomerella*) found on camellia is morphologically similar to the one causing azalea anthracnose; however, isolates from camellia failed to cause infection in azaleas. Since camellias are often grown in close association with azaleas, it perhaps is reasonable to assume that a specialized race of the fungus particularly pathogenic to azaleas arose in some way from the camellia pathogen. Some observational evidence may be offered in support of this speculation. Although azaleas are grown widely in Louisiana and *G. cingulata* occurs on many hosts everywhere, the epidemics of a severe form of the disease occurred only in a small area in the state. Of several isolates of the fungus from azaleas in New Orleans and Lecompte (areas in which the disease is mild and infrequent), only 1 proved pathogenic, whereas all the isolates from the Folsom-Loranger area (where the disease is severe) were found to be pathogenic. It may not be unreasonable, therefore, to assume that a race pathogenic to azaleas has, in some manner, arisen in this particular area.

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[7] Peterson, D. H. 1953. Anthracnose of peach. Yearbook Agr., U. S. Dept. Agr. p. 993-694.

REPORT OF THE 1957 ANNUAL MEETING OF THE NORTHEASTERN DIVISION OF THE AMERICAN PHYTOPATHOLOGICAL SOCIETY

The eighteenth annual meeting of the Northeastern Division of the American Phytopathological Society was held at West Springfield, Massachusetts, November 7 and 8, 1957. About 160 people were in attendance. Thirty scientific papers were presented, and several informal progress reports on results of 1957 fungicide tests were made.

Officers elected for 1958 were as follows: *President*, B. A. FRIEDMAN; *Vice-President*, A. E. RICH; and *Secretary-Treasurer*, J. E. LIVINGSTON. D. S. WELCH continues as Councilor for one more year.

AVERY E. RICH, *Secretary-Treasurer*

Transmission of the dapple apple virus. BARRAT, J. G., W. W. SMITH, AND A. E. RICH. Symptomless 15-year-old apple trees of Cortland, McIntosh, Golden Delicious, Starking Delicious, Baldwin, and Macoun varieties were inoculated by budding in the spring of 1955. The source of inoculum was a tree topworked to Cortland with an interpiece of Virginia Crab, both showing symptoms of dapple apple. Three or 4 buds from either the Cortland variety or the Virginia Crab interpiece were placed in each of 2 branches on opposite sides of each tree. Symptoms first appeared in 1957 on Cortland, McIntosh, Golden Delicious, and Starking Delicious. Apples in the vicinity of the inoculating buds in either direction along the limb remained symptomless; however, those growing on the first or second side branches centripetally to the buds developed symptoms, indicating an unusual pattern of virus movement through the tree. A Starking Delicious tree with 10 buds placed at random had a greater number of dappled fruits than did McIntosh or Cortland budded on opposite sides. An incubation period of more than 1 growing season appears to be necessary for expression of symptoms.

Influence of pH, nutrition, and soil moisture on the development of large brown patch. BLOOM, J. R., AND H. B. COUCH. A continuous-flow, drip-culture system, with silica sand as the supporting medium, was used for growing Seaside bentgrass (*Agrostis palustris*) at various levels of nutrition and pH. Postemergence inoculation with *Rhizoctonia solani* was accomplished by the addition of aqueous mycelial suspensions around the crowns of the plants. Disease severity was much greater when plants were grown at low nitrogen fertility than when grown at normal levels (odds 99:1). High nitrogen nutrition resulted in lower disease incidence than did normal nitrogen nutrition (odds 19:1). High or low levels of phosphorous or of potassium did not influence disease reaction; however, when the plants were grown at a low level of balanced nutrition, disease severity was greater than when plants were grown at a normal level (odds 99:1). At low nitrogen levels, alteration of pH did not influence disease reaction. At high nitrogen levels, however, disease intensity was least at pH 4.0 and at pH 7.0 (odds 99:1). The influence on disease development of 5 soil-moisture levels, ranging from field capacity to permanent wilting percentage, was checked by varying the time of irrigation prior to inoculation at each level. Within this soil-moisture range, no differences in disease severity were noted.

Effect of sterile filtrates of cultures of Pseudomonas marginalis upon lettuce tissue. CEPONIS, M. J., AND B. A. FRIEDMAN. Vein browning and russet spotting, previously reported as part of the brown spot complex, have been observed on lettuce tissue distant from the site of active natural infections by *Pseudomonas marginalis*, the cause of bacterial soft rot. Tissues showing these symptoms were usually free from bacteria. Vein browning, russet spotting, and soft rot maceration of tissues have been induced in Romaine and Iceberg-type lettuce with sterile cell-free filtrates of 3- to 4-day-old cultures of *P. marginalis* grown in lettuce broth. Symptoms appeared within 17 hours on Romaine lettuce leaves standing at room temperature in 50 ml of the filtrate to which 100 ppm oxytetracycline had been added. Reduced pressure was applied initially to facilitate uptake of the filtrate. Lettuce leaves dipped in the filtrate and then incubated for 18 hours in a moist chamber developed severe russet spotting. These symptoms failed to develop on leaves treated with autoclaved filtrates or with sterile lettuce broth. Studies are under way to determine the nature of the enzymes or other active agents in the sterile culture filtrates of *P. marginalis*.

Effects of fungicidal and combination sprays on apple finish and on mite populations. DAINES, ROBERT H. A 2-year study indicated that the best finish, in decreasing order, on Golden Delicious apples was obtained with the following: captan plus lead arsenate; captan plus methoxychlor; thiram plus lead arsenate; thiram plus dieldrin; captan plus dieldrin; glyodin plus lead arsenate; glyodin plus dieldrin; glyodin plus methoxychlor. Where glyodin was used in the prebloom and third and fourth cover sprays, where glyodin was used in the third and fourth covers and captan in all remaining sprays, or where sulfur was used alone or with captan in the precover sprays and captan in remaining sprays, the finish was as good as with any combination tested. Red Delicious apples developed a fine finish from all treatments except where sulfur was used early. Fruit on the sulfur treatments developed considerable russeting of cheek and stem end and frost ring russet. Ovex (4-chlorophenyl-4-chlorobenzene sulfonate) was applied in all petal fall and first cover applications. Mite counts per leaf on June 28 were as follows: glyodin, 3; captan in all sprays except glyodin in prebloom spray and in third and fourth cover sprays, 4; captan in all sprays except glyodin in third and fourth cover sprays, 10; thiram, 12; captan, 35; and sulfur early followed by captan, 70. In a Stayman block, where all treatments received ovex at pink and petal fall, mite populations in ascending order were as follows: Thioneb (50% polyethylene thiuram sulfide), glyodin, Cyprex (70% *n*-dodecylguanidine acetate), thiram, zineb, captan, dichlone, and sulfur.

Cell wall structure and composition in the conidiophores of Aspergillus niger and A. carbonarius. FARR, WANDA K., AND J. E. LIVINGSTON. Examination of the cell walls of *Aspergillus niger* and of *A. carbonarius* showed a microscopically structureless primary layer and secondary deposits of fibrils in spiral arrangement. Observations in plane-polarized light showed that the primary layer is composed of isotropic material and that the secondary layers contain anisotropic material oriented parallel to the main axes of the constituent fibrils. Deposition of material forming the secondary lamellae progresses from the base to the tip of the conidiophore. Cytochemical and X-ray diffraction analyses revealed the chitinous nature of the primary layer of the conidiophore and the cellulosic nature of the fibrillar secondary deposits. Cytochemical reactions of the secondary layers for protein, pectic material, and callose were positive. Certain differences in the reactions of the primary wall layers of *A. niger* and of *A. carbonarius* appeared to be due to the presence of fatty acid in the wall of *A. carbonarius* in addition to the chitin.

Bacterial fleck of cabbage. FRIEDMAN, B. A., S. M. RINGEL, AND M. J. JAFFE. Leaf spotting of cabbage and of Chinese cabbage occurs regularly in various sections of the United States, occasionally causing serious market losses. The spots are fleck-like, circular to elongate, and gray, brown, or black in color; they generally are scattered over both surfaces of the outer or inner leaves of the heads. Lesions are restricted to epidermal cells and to a few of the underlying parenchymal cells. Leaf-spotting fungi were not found in affected tissue. F. O. Holmes, Rockefeller Institute for Medical Research, was unable to isolate the turnip mosaic virus from 4 lots of cabbage showing fleck symptoms. The disease was reproduced on normal leaf blades at 35° and 70°F with water suspensions of 6 species of bacteria isolated from natural infections and identified as *Pseudomonas* sp., *P. maculicola*, *P. fluorescens*, and 3 species of *Bacterium*. Droplets of rain, dew, or moisture of condensation may occur on cabbage leaves in the field, in transit, or in storage; this apparently provides opportunity for various types of bacteria to induce cabbage fleck under widely varying conditions.

Potato seed-piece treatments. FINK, HARRY C. Results from 3 years' experiments with potato seed-piece treatments indicated that combinations of fungicides and streptomycin sulfate may result in stands and yields significantly lower than those obtained when no treatments are used. Inclusion of a third pesticide in the combination may add to or negate the ill effects. Eleven fungicides were tested alone and in combination with streptomycin sulfate or with dieldrin and streptomycin sulfate. Seed piece rot due to *Erwinia atroseptica* was controlled with streptomycin sulfate (100 ppm) in laboratory experiments. Sprout lesions caused by *Rhizoctonia solani* also were controlled in the laboratory by certain fungicides alone or in combination with streptomycin sulfate or with streptomycin sulfate and dieldrin. This control of rots was not obtained in the field when the seed pieces were dipped in solutions of the pesticides, allowed to dry, placed in open rows in Hagerstown silt loam, and inoculated with a spray containing *E. atroseptica* before covering. No significant differences in stand or yield resulted from any of the treatments. Precipitation was normal the first year, extremely high the second, and extremely low the third. Temperatures were near normal all 3 years. Under these conditions, no statistically significant increases in stand or yield were realized, but statistically significant decreases were found where combinations of pesticides were used.

Bioassay test for the detection of pentachloronitrobenzene. GASIORKIEWICZ, E. C. In phytocidal and fungicidal tests with pentachloronitrobenzene (PCNB), inhibition of the common wood sorrel (*Oxalis repens* Thunb.) was noted. Oxalis was controlled with PCNB (54 g and 20% active ingredient per gal. of water) when applied as a soil drench at a rate of 1 pint per sq. ft. Weed control of Oxalis was of a postemergence type in that seeds germinated in the soils but the seedlings died as a result of a shriveling at the soil line. Germination and subsequent killing of seedlings in benches continued for 8 months following 2 applications made 2 months apart. Steam pasteurization had no immediate effect on treated soils. Following steam pasteurization and under normal watering practices, Oxalis weeds were controlled for an additional 3 months without reapplication of the chemical. *O. repens* was found to be a diagnostic bioassay test plant for determining the persistence of PCNB in treated soils.

Storage rots of carnations. GASIORKIEWICZ, E. C. Low-temperature conditioning of cut flowers and of rooted and unrooted carnation (*Dianthus caryophyllus* L.) cuttings at 31°F is a recent cultural development. Holding periods vary from a few days to 4 weeks for flowers and from a few days to 6 months for cuttings. During low-temperature conditioning, a storage rot of flowers was found to be caused by *Botrytis cinerea* Pers. This disease was characterized by

a water-soaked flecking of the oldest outer petals, followed by browning, matting of petals, and sporulation of the fungus. Storage rots on leaves and stems of carnation cuttings occurred in the cold-temperature rooms and in propagation benches, with or without mist. Storage leaf rot, caused by *Alternaria dianthi* F. L. Stev. & Hall, developed distinct lesions with purple margins, followed by a leaf necrosis and sporulation. Symptoms of stem rot, caused by *B. cinerea*, usually appeared as a mycelial growth on the leaf, followed by a rot of the infected tissue; sporulation rarely occurred at 31°F but occurred readily at 40°F and above. Fungicidal sprays or dips with captan or zineb have effectively controlled storage rots on cuttings.

A comparison of apple mosaic virus isolates. GILMER, R. M. Five isolates of viruses causing mosaic of apple were compared, and except for minor differences in incubation period in apple seedlings and in symptoms, 3 isolates from New York and 1 from Washington were similar. None of these 4 isolates was transmitted mechanically or by dodder from apple foliage, but each was transmitted to apple and flowering quince by bud inoculation. They were not transmitted by budding to pear, common quince, hawthorn, or *Sorbus aucuparia*. An isolate from California was markedly different from the other isolates. This virus was transmitted by dodder from apple to apple, from apple to tobacco, and from tobacco to apple; it was mechanically transmitted from apple foliage to cucumber, tobacco, pepper, bean, and *Tithonia speciosa*. The same virus was transmitted by bud inoculation to quince, flowering quince, pear, hawthorn, and *S. aucuparia*. It was recovered after introduction into apple seedlings already systemically infected by the 4 other isolates of apple mosaic virus. Tobacco plants systemically invaded by the California virus were not protected against infection by tobacco streak virus or against reinfection by the California virus itself.

Insect transmissions of X-disease virus. GILMER, R. M., AND F. L. MCEWEN. A technique for rapid transmission of X-disease virus with leafhopper vectors has been developed. X-disease virus was initially transmitted from infected chokecherry seedlings to seedlings of periwinkle (*Vinca rosea*). Infected periwinkle plants showed marked floral and foliar symptoms and remained acceptable to many possible vector species for long periods of time. Suspected vectors were permitted a 5- to 10-day acquisition period on infected plants, transferred to healthy periwinkle seedlings, and allowed to feed for 25 days. Under these conditions, X-disease virus was transmitted by *Fieberiella florii*, *Scaphytopius acutus*, and *Gyponana striata*. Symptoms appeared in 4–6 weeks. X-dicase virus was transmitted from chokecherry to periwinkle, from periwinkle to periwinkle, and from periwinkle to peach by nymphs and adults of *F. florii*. Nymphs of this species also transmitted the virus to peach from periwinkle plants originally infected by means of dodder transmission. Attempts to transmit X-disease virus to aster seedlings with *F. florii*, *Macrosteles fascifrons*, and dodder were unsuccessful.

The influence of timing and rates of usage of Karathane on the control of apple powdery mildew. GROVES, A. B. Karathane (22.5% 2-(1-methylheptyl)-4,6-dinitrophenyl crotonate and 2.5% isomers) was applied to the susceptible Rome variety at rates of 4, 8, 12, and 16 oz. per 100 gal. of spray at 7-, 14-, and 21-day intervals throughout the period of infection by *Podosphaera leucotricha* (Ell. & Ev.) Salm. Treatments were applied separately from a standard spray program. Applications of Karathane began in the late bloom period and continued at the noted time intervals until terminal growth ceased. Foliar infection counts were made following completion of 9 weekly applications. The scoring method was based on a count of the actual number of infections per leaf sample, according to the method reported before this group in 1956. Percentage control of infection by application of 16, 12, 8, and 4 oz., at weekly intervals was 99.7, 99.0, 98.4, and 94.5, respectively; at 2-week intervals it was 95.9, 93.5, 87.0, and 86.5, respectively; and at 3-week intervals it was 85.4, 86.7, 75.0, and 58.4, respectively. The break in dosage response came between 8 and 12 oz.; the break in influence of timing interval came between 7 and 14 days.

Control of Coccomyces hiemalis by systemic movement of cycloheximide semicarbazone in sour cherry following root or leaf absorption. HAMILTON, JAMES M., AND MICHAEL SZKOLNIK. Soil application of the semicarbazone derivative of cycloheximide provided systemic activity against the cherry leaf spot fungus (*Coccomyces hiemalis*) in 3-year-old potted Montmorency and English Morello cherries. A single soil application of 20 ppm of the semicarbazone (200 ml per 7-in. pot), or an application of 5 ppm on 2 consecutive days, effectively protected the foliage against infection when inoculated on the third day following start of soil treatment. Subsequent inoculation 2 weeks later, after 8 new leaves had developed, resulted in no infection. Concentrations of 100 ppm or higher resulted in short internodes and injury to the foliage. Cycloheximide was less effective and more injurious than were the derivatives at equivalent concentrations. The systemic movement and activity of the semicarbazone derivative, when applied to specific individual cherry leaves on terminals, was correlated with the concentration used, with rate of tree growth, and with age of leaves. Such movement and activity were more pronounced on trees grown in pots outdoors than on those grown in the greenhouse. Semicarbazone (20 ppm) did not move in appreciable amounts from a given sprayed leaf to the next distal leaf but was translocated to the next leaf in vascular alignment on the stem. Thus, multiple leaf spraying was essential for the systemic protection of the new growth.

Movement of dodecyl guanidine derivatives through the leaf in the control of apple scab and cedar-apple rust fungi. HAMILTON, JAMES M., AND MICHAEL SZKOLNIK. Foliage of greenhouse-grown Rome beauty apple trees was protected against the apple scab fungus (*Venturia inaequalis*) and the cedar-apple rust fungus (*Gymnosporangium juniperi-virginianae*) by spraying the underside of the leaf with the acid phthalate and with the acetate salts of dodecylguanidine (American Cyanamid experimental fungicides 23441 and 5223, respectively) prior to inoculating on of the top side of the leaf with the respective fungi. The fungicides were evaluated on the basis of ⅓ and 1 lb. active chemical per 100 gal. with the addition of a surface-active material (Tween 20). These materials were ineffective when applied in this manner in postinfection tests. Local systemic protective inhibition or suppression of scab was also obtained with captan, dichlone, glyodin, and ferbam, although such activity was not as effective and consistent as that of the dodecyl guanidines. This activity with the standard fungicides was observed, for the most part, on terminals that were growing slowly, and the leaves were not very susceptible to scab.

The mechanism of plant virus inoculation. HILDEBRAND, E. M. Use of a micropipette to make cell punctures simulating those made by an insect vector and thus to inoculate plants with viruses was perfected in 1939. The micropipette, simulating an insect proboscis and ranging in size from 1 to 5 μ, was operated by a micromanipulator under a compound microscope. Trichomes of Bonny Best tomatoes were thus successfully inoculated with aucuba mosaic virus. The micrurgical instrument, loaded with virus, perforates the trichome wall. Protoplasm is extruded quickly, forming a hemispherical droplet that bathes the pipette tip momentarily, and then is drawn back into the cell carrying along some of the virus. This pulsing action takes place consistently in about 0.6 second, apparently because of the application and release of pressure, and is characteristic of all safe mechanical inoculation techniques tried. In order to survive, the inoculated cells must plug the wounds in-

stantly and resume protoplasmic streaming. Essentially obligate wound parasites, viruses multiply inside living cells and diffuse through protoplasmic strands into adjoining cells. Wounding and virus entry appear coincidental, since any remaining inoculum can be washed off without detriment to infection.

Distribution of sexual-compatibility strains of Ceratocystis ulmi in Massachusetts. HOLMES, F. W. Cultures of *Ceratocystis ulmi* (Buis.) Moreau were grown adjacent to compatibility strains "A" and "B" of *C. ulmi* on autoclaved elm wood slices. Perithecia formed within 1–3 months. Cultures have been tested from 112 towns in 11 counties of the state. All except 6 formed perithecia when grown with "B." Cultures from Wrentham, Seekonk, Duxbury, Hingham, Nahant, and Manchester formed perithecia when grown with "A." No cultures formed perithecia with both "A" and "B." It seems likely that distribution of strain "B" may be limited to eastern Massachusetts and therefore that genetic recombination in this heterothallic fungus cannot occur in the western part of the state. In that case, new pathogenic strains that are able to kill elms resistant to the Dutch elm disease should not arise quickly in this area after resistant trees are introduced.

Production of cellulase by Fusarium oxysporum f. lycopersici. HUSAIN, AKHTAR, AND A. E. DIMOND. Culture filtrates of *Fusarium oxysporum* f. *lycopersici* showed high cellulase (CX) activity when the fungus was grown on Richard's solution containing cellulose as a carbon source but not when glucose was the carbon source. Cellulase activity was high when the carbon source was a 0.5% solution of carboxymethyl cellulose (CMC-70 high and CMC-30 low), autoclaved tomato stem sections, or filter paper cellulose. The fungus was grown on living tomato stem sections sterilized by propylene oxide vapors to investigate the production of cellulase on living tissue. The CX enzyme was produced on inoculated stem sections, but no enzyme was detected in noninoculated stem pieces. A solution of partially purified enzyme caused wilting of tomato cuttings when the enzyme solution was introduced through cut ends of the stems for 16–24 hours. No injury was caused by a heat-inactivated enzyme sample. These results indicate that the fungus is a cellulolytic organism and suggest that cellulase produced by this pathogen may play a role in the disease syndrome.

Cellulolytic activity of some apple-rotting fungi. HUSAIN, AKHTAR, AND A. E. DIMOND. Three apple-rotting fungi, *Botryosphaeria ribis*, *Glomerella cingulata*, and *Physalospora obtusa*, were examined for ability to produce cellulase in culture. The fungi were grown on a synthetic medium containing ammonium nitrate and filter paper, wood cellulose, carboxymethylcellulose (CMC-30), or glucose as sources of carbon. Filtrates of week-old cultures were assayed for cellulase activity by estimating loss in viscosity of a 0.5% CMC solution (CMC-70 high) and by determining the increase in reducing sugar (by Nelson's modification of Somogyi's method) in a 0.5% CMC solution (CMC-30 low). All fungi produced high cellulase activity in cellulose media but little or none in glucose media. All fungi caused disintegration of filter paper strips within 2 weeks, indicating the presence of a cellulase capable of attacking native cellulose. Available data indicate that these fungi possess enzyme systems that can degrade cellulose and that may also play a role in disintegration of plant tissue by the pathogens. Cellulase production by these fungi may be important also in their ability to form cankers on woody parts of the plants.

Cytopathogenic effects of the aster yellows virus on its insect vector. LITTAU, VIRGINIA C., AND KARL MARAMOROSCH. Our earlier report that the fat body cells of the vector of aster yellows virus, *Macrosteles fascifrons* Stål, undergo characteristic changes after the vector has acquired virus by feeding on diseased plants has been confirmed. The nuclei become stellate; basophilic cytoplasmic material becomes stringy and sparse and apparently is easily torn. Cell boundaries are not clearly seen (Carnoy fixation, azure B staining). In contrast, nuclei of fat body cells of nonviruliferous insects tend to be round or to possess smooth contours. The cytoplasm is generally homogeneous, with numerous vacuoles of varied size enclosed with a definite cell membrane. Virus-induced changes are seen better in males than in females. Cytopathogenic changes occur after the insects have fed on diseased plants for 18–28 days. Other insects that had been injected with the juice of viruliferous insects and allowed to feed on immune rye plants or on healthy asters for 28 days also developed typical changes in the fat body. These results indicate that this plant virus causes a disease in its animal vector.

Chemical reactions involved in the fungitoxicity of captan. LUKENS, R. J., AND H. D. SISLER. Captan (*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide) reacts with sulfhydryl compounds such as cysteine. The reaction with cysteine produces tetrahydrophthalimide, cystine, H_2S, CS_2, 2-thiazolidinethione-4-carboxylic acid ($C_4H_5O_2NS_2$), and HCl. The formation of $C_4H_5O_2NS_2$ demonstrates that thiophosgene is produced as an intermediate. $C_4H_5O_2NS_2$ is also produced from cysteine plus thiophosgene or $ClSCCl_3$. The CS_2 and H_2S arise from the reaction of thiophosgene with water. Evolution of CS_2 from yeast cultures treated with captan indicates the presence of thiophosgene *in vivo*. In contrast to captan, thiophosgene will react with serine, histidine, and possibly other amino acids. $ClSCCl_3$ is nearly as toxic as is captan to *Saccharomyces pastorianus* Hans., thiophosgene is somewhat less toxic, and tetrahydrophthalimide is nontoxic. The imide portion of captan appears to stabilize the $-SCCl_3$ toxiphore and to modify the penetration and reactivity of this group. The fungitoxicity of captan may arise from either or both of 2 sources: 1) $-SCCl_3$ may combine through its sulfur with certain vital cellular constituents, and 2) $-SCCl_3$ will react with sulfhydryl groups to release thiophosgene, which can combine with $-SH$, $-NH_2$ or $-OH$ groups within the cell.

Small field plots for experiments involving plant pathogenic nematodes. MAI, W. F. It is often difficult to locate uniformly infested areas or to prevent transfer of nematodes between plots after treatment when naturally infested fields are utilized for tests with plant pathogenic nematodes. For the past 10 years, 5 × 8-ft. plots bounded by 12-in. redwood boards and separated by alleys at least 2 ft. wide, have been used successfully for certain types of research involving the golden nematode, *Heterodera rostochiensis* Wr. A tractor-mounted shovel was used to mix infested soil thoroughly, to place it in plots, and to remove it from plots after an experiment. All cultural operations and observations were made by an operator standing in the alley, thus eliminating the possibility of transporting nematodes from one plot to another on footwear. Tools were cleaned with water and paper towels before use in each plot. Measured quantities of water were applied with a portable irrigation unit consisting of elevated perforated copper pipes and a small water meter. Plots may be sprayed with a tractor-drawn unit if sufficient roadways are left. Reliable estimates of potato yields can be made from 5–6 plots. Plant spacings corresponding to those in large fields permit typical root development and nematode increase.

Attempted chemotherapy of plant viruses in insect vectors. MARAMOROSCH, KARL. A benzimidazole riboside (DRB) that was shown by Mirsky et al to inhibit ribonucleic acid synthesis and by Tamm et al to inhibit influenza virus multiplication in tissue culture was tested *in vivo* against 4 plant viruses in their respective insect vectors. Aster yellows, curly top, corn stunt, or wound tumor virus was acquired by leafhopper vectors feeding on diseased plants. A suspension of 5 mg DRB/ml, 0.001 ml per insect, was injected after virus acquisition. An increase in the

length of virus incubation periods or an inhibition in virus transmission was used as the criterion of inhibition of virus multiplication. The results of extensive tests indicated that DRB had no effect on the multiplication of the 4 plant viruses in leafhoppers.

Superior control of tobacco stunt nematodes with a nematocide mixture. MILLER, P. M., AND G. S. TAYLOR. In pests fumigated with 12 gal./acre of Dorlone (a mixture containing 19% ethylene dibromide (EDB) and 75 per cent 1,3-dichloropropene) the average plant height of shade tobacco was 84% greater than that of plants in untreated plots. This difference in average plant height was greater than that provided by 16 gal./acre of Dorlone or by treatment with either of the 2 active ingredients of Dorlone, 16 or 20 gal./acre of Telone (technical 1,3-dichloropropene) or 4.5 gal./acre of Dowfume W-85 (85 per cent EDB). Tobacco grown in this same field was severely stunted in 1956 by heavy infestations of the tobacco stunt nematode, *Tylenchorhynchus claytoni*. Soil tests showed little difference in fertility among plots. Greenhouse tests indicated uniformly low populations of root-rotting organisms. Differences between treatments, therefore, apparently were caused by nematode control during early plant growth, even though stunt nematode populations were low 6 weeks after planting. (This period was one of high soil temperatures and low soil moisture.) A combination of nematocides improved control where 1 type of nematode predominated. Improved control of mixed nematode populations with nematocide combinations is indicated.

Verticillium wilt of broccoli and cauliflower in New York. NATTI, JOHN J. Seedlings of 15 varieties of broccoli and of 3 of cauliflower were found susceptible to infection in greenhouse tests by isolates of *Verticillium albo-atrum* Reinke & Bert. from tomato. Plants were inoculated by dipping roots of 10-day-old seedlings in a homogenized mixture of mycelium and potato-dextrose agar, the medium upon which the fungus was grown. Seedlings were then planted in pasteurized soil. Some plants developed symptoms within 14 days whereas others began to show symptoms about 30 days after inoculation. Symptoms consisted of yellowing of the lower leaves and a black discoloration of vascular strands. Frequently only 1 side or a portion of 1 side of the leaf was yellow. A common symptom resembling that of black rot was yellowing of the leaf tip or of wedge-shaped areas of the leaf margins. Broccoli and cauliflower plants, grown in field plots known to be infested with *V. albo-atrum*, developed symptoms identical to those of experimentally inoculated plants. Infected plants were not stunted and yields did not appear to be affected. Broccoli plants inoculated in the greenhouse with isolates from naturally infected broccoli and cauliflower plants developed symptoms identical to those incited by isolates from tomato plants.

Purple blight—an undescribed disorder of Pisum sativum. SCHROEDER, W. T., N. H. PECK, AND M. T. VITTUM. Pea plants affected in the field first appear stiffly erect with sparse foliation. Brownish flecking is apparent on the stipules and leaves, is barely visible on the young foliage, but increases in intensity and in purple coloration until it culminates in a purplish-black desiccation of the oldest leaves. Stems and tendrils of affected plants may show brownish blotches, but roots are apparently unaffected and remain normal in appearance. Purple blight may develop on plants at any stage of growth, but injury is greatest when the disorder occurs early and results in a blighted plant with no pods or very few, small pods. Affected plants have never been observed to recover or to resume normal growth. Although purple blight has occurred in 10 of the 14 years since it was first observed, the disorder is sporadic in occurrence and may not develop in the same area 2 years in succession. Experiments with soils from affected and nonaffected portions of a field suggest that purple blight may be of nutritional origin, possibly resulting from the toxic interactions of manganese, iron, and aluminum.

Evidence for carbon dioxide fixation by uredospores of the bean rust fungus. STAPLES, RICHARD C., AND LEONARD H. WEINSTEIN. Uredospores of the bean rust fungus, *Uromyces phaseoli*, were obtained from greenhouse-grown Pinto bean leaves. Spores washed with sterile water were incubated for 4 hours with 100 μc $NaHC^{14}O_3$ in a closed system. Labeled carbon was found in amino and other organic acids as determined by a thin-window Geiger-Müller tube and by autoradiographic technique. Intermediates of the Krebs cycle found to be labeled included succinic, fumaric, citric, and malic acids. Malic acid was not detected in spores not treated with bicarbonate. Labeled amino acids found included glutamic acid, aspartic acid, serine, glycine, γ-aminobutyric acid, alanine, glutamine, and asparagine. Thus, it appears that uredospores of *U. phaseoli* contain an active dark $C^{14}O_2$ fixation mechanism, presumably via the PEP carboxylase system. The results also suggest the participation of an organic acid cycle, with active transamination reactions into alanine, glutamate, and aspartate.

TITERS OF THREE NONPERSISTENT APHID-BORNE VIRUSES AFFECTING PEPPER IN SOUTH FLORIDA[1]

John N. Simons[2]

SUMMARY

Titers of active virus of 3 pepper viruses (veinbanding mosaic virus and 2 strains of cucumber mosaic virus—southern cucumber mosaic virus and pepper calico virus) were measured in pepper by means of transmissions by the green peach aphid, *Myzus persicae* (Sulz.), and by sap inoculations. In general, a close correlation was found between numbers of transmissions resulting from the feeding of single aphids and from rubbing test plants with sap diluted 1:500. Titer of all 3 viruses reached maxima at 2 weeks from time of inoculations. In the case of the cucumber mosaic strains, the titer dropped rapidly and remained low from 3 weeks after inoculation until the end of the tests, 4 weeks later. The concentration of veinbanding mosaic virus remained high and constant from 2 weeks after inoculation to the termination of the trials. Curves of the virus titer in weed hosts were found to be similar to those found in pepper.

Studies with double infections indicated that a net decrease in virus titer of each virus of a pair resulted regardless of the order of inoculation with the 2 viruses.

Previous work (10, 12) has reported large differences in rates of spread among the 3 common aphid-borne viruses of pepper in the Everglades area of south Florida. Veinbanding mosaic virus (VBM), probably potato virus Y (10), spreads much more rapidly than does southern cucumber mosaic virus (SCuM), whereas pepper calico virus (PCV), also a strain of cucumber mosaic virus (12), occurs only rarely. Although differences in relative transmissions among the 3 viruses are believed important in explaining this phenomenon (10, 12), it seems likely that other factors also influence the rates of field spread.

Since most of the total spread occurs as the within-field type (11) and because the viruses are all similar in their vector-virus relationships (10, 12), the most logical area of exploration would seem to be the host plant-virus relationships in pepper.

MATERIALS AND METHODS.—Viruses were maintained in pepper (*Capsicum frutescens* L. var. California Wonder) plants that were grown in an air-conditioned greenhouse in which the temperature ranged from 70° to 80° F. Pepper seedlings in the 2- to 4-leaf stage were used as test plants.

Noninfective green peach aphids were reared on healthy caged pepper plants. Late instar or adult apterae were used as vectors. Aphids were starved from 1 to 2 hours before being given watched and timed acquisition feeding periods of 15 to 20 seconds duration. Test feeding times ranged from 1 to 2 hours; 1 aphid was used per test plant.

The assay procedures used with aphid feeding were as follows. Inoculum sources were prepared by rubbing the lower and middle leaves of several pepper plants in the 8- to 10-leaf stage with each of the 3 viruses. Five plants for each virus were set aside for use as inoculum sources for trials with aphids. At 1, 2, 3, 4, 5, and 7 weeks after inoculation, aphids were given opportunity to feed on leaves from 3 general areas of each plant as follows: 1) lower, 2) middle, and 3) upper leaves. Ten aphids were fed on each of the 5 source plants, making a total of 50 aphids used for each virus each week.

Concurrent with the aphid trials, mechanical inoculations tests for titer of active virus also were carried out. Two 1-ml samples of freshly expressed sap from leaves of plants similar to those used as aphid inoculum sources were diluted 1:500 and 1:5000 with tap water. Test plants, lightly dusted with 500-mesh Carborundum, were inoculated by means of cotton swabs rubbed twice on each of 2 leaves and once on each cotyledon. These plants were immediately rinsed with tap water. Twenty-five plants were inoculated by rubbing with each of the 2 samples (50 plants in all) of each of the 3 viruses. The entire experiment was repeated, making a total of 100 plants inoculated with each virus each week of infection both by aphids and by rubbing.

All plants were sprayed routinely with nicotine sulfate to control insects in the greenhouse.

RESULTS.—*Titers of VBM, SCuM, and PCV in pepper.*—Except at the end of the first week for SCuM and PCV, the results obtained with aphid transmissions closely paralleled those obtained by rubbing test plants with sap diluted 1:500 (Fig. 1, A–C). Evidently both techniques measured relative concentrations of active virus. The differences in relative aphid transmissibility of the 3 viruses 2 weeks after inoculation were similar to those obtained before (10, 12).

The large discrepancy between numbers of aphid transmissions and numbers of mechanical transmissions 1 week after inoculation with SCuM or PCV was investigated further. Since the short (15–20 seconds) aphid feedings sampled virus from the epidermis (4) and the expressed sap contained virus from the entire leaf, one possible explanation of the results was that

[1] Accepted for publication December 20, 1957.

Florida Agricultural Experiment Station Journal Series No. 696.

[2] The author wishes to express his appreciation to Mr. E. King for preparing the graphs and to Mr. Henry Spelman III for making the photographs.

early in the infection there was more virus in mesophyll tissue than later. Several attempts were made to verify this hypothesis by the use of both short (15–20 seconds) and longer (5–10 minutes) acquisition feedings on plants infected with SCuM for 3–6 days. In all cases, only limited transmission was obtained with either short or long acquisition feedings. Although these findings apparently refute the hypothesis, recent evidence (4) has shown that the depth of stylet penetration (which increases with length of acquisition feeding time), may influence the probability of an aphid's becoming infective.

Another approach to this anomaly was to work with VBM. Titers of VBM at 1 week after inoculation were similar with both inoculation techniques, thus it was decided to sample plants at periods during the first week. It was thought probable that VBM multiplies faster than do the cucumber mosaic strains (Fig. 1, A–C); thus it seemed possible that a discrepancy between virus titers obtained by the 2 methods might be demonstrable if the sampling date were earlier than 1 week. Two trials that compared the 2 assay methods at 4, 7, and 14 days after inoculation were carried out. Differences between aphid and sap transmissibility of VBM at an early stage of infection were similar to those found for SCuM and PCV (Table 1). Differential concentrations of virus in mesophyll and epidermis still seem a logical explanation of the phenomenon.

TABLE 1—*Comparison of transmission of veinbanding mosaic virus in pepper by aphids and by rubbing test plants with a 1:500 dilution of sap*

Age of inoculum plants in days	Percentage transmission[a]	
	Aphid	1:500 sap
4	3	14
7	44	60
14	67	66

[a] Combined results of 2 trials, each with 50 plants for each assay on each date.

Because of the low transmission of SCuM and PCV obtained during most of the experiment, evidence on distribution of virus in the plant was too sketchy to be useful. Titer of VBM remained high, however, and results indicated that there was not much difference among the 3 areas sampled as to virus concentration.

Titers of VBM and SCuM in weed hosts.—Since it is known that nightshade, *Solanum gracile* Link, is an important weed host of VBM (10) and that wandering Jew, *Commelina nudiflora*, harbors SCuM (5), it was thought desirable to check by aphid-transmission the titers of active virus in these species. Procedures were similar to those used for the aphid work in pepper. Results with VBM in nightshade were as follows: 1 week,

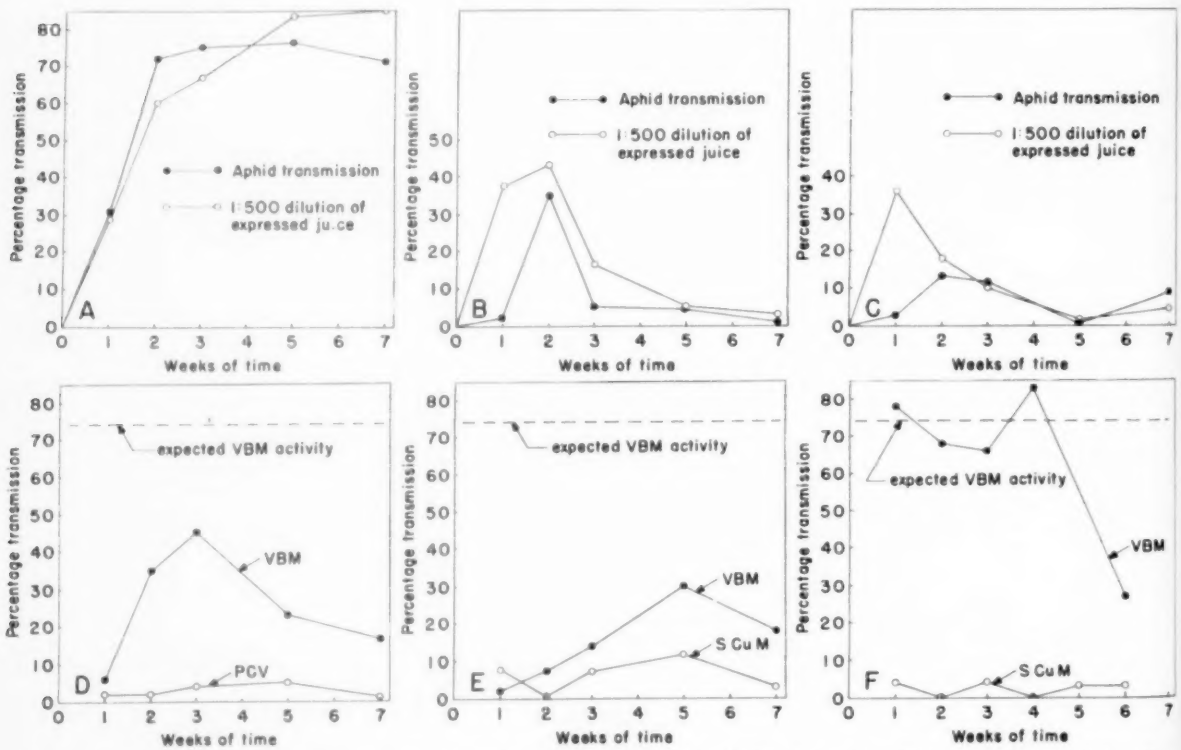

FIG. 1. A–C) Comparison of percentage transmission obtained with aphid and sap inoculations with A) veinbanding mosaic virus (VBM), B) southern cucumber mosaic virus (SCuM), and C) pepper calico virus (PCV). D–F) Virus titers, as determined by percentage aphid transmission, for D) PCV-infected plants inoculated with VBM, E) SCuM-infected plants inoculated with VBM, and F) VBM-infected plants inoculated with SCuM.

no sample; 2 weeks, 10 per cent transmission; 3 weeks, 60 per cent; 4 weeks, 60 per cent; and 6 weeks, 58 per cent. A maximum in virus activity was reached in 3 weeks instead of in 2 as in pepper, but the same general type of curve was obtained in both species. In the case of SCuM in wandering Jew, transmission ranged from 0 to 5 per cent over the 6-week period of sampling, with no maximum in activity indicated. Neither plant species seemed to be a better source of the respective viruses than was pepper.

Titers in pepper with 2 simultaneous infections.—Because it is not unusual to find mixed infections involving SCuM and VBM in pepper fields, and because the virus activity curves of the 2 viruses were so different, it was decided to study titers of the viruses under conditions of double infection. Studies were made with the following combinations in pepper: 1) VBM superimposed on SCuM; 2) VBM superimposed on PCV; 3) SCuM superimposed on VBM; and 4) SCuM superimposed on PCV.

The same plants, or similarly treated ones, that had been used for the aphid-assays of single infections were reinoculated by rubbing them with a second virus. These inoculum source plants were some 2 months older than they were when used for the single-infection assays; conceivably, this difference in age of the plants could result in different virus activity curves. Early work with VBM had indicated that young pepper plants (2-leaf stage) did not make as potent a virus source as did older ones, but once the plant had reached the 8- to 10-leaf stage, initial virus buildup was similar regardless of whether the plant was 6 weeks or 12 weeks old. It was thought desirable to use plants in which the first infection had become well established. Thus it was decided to use the same series of inoculum plants for all tests. They were once more assayed by aphids in a manner similar to that used for the single-infection trials. The experiment was repeated so that a total of 100 aphids were fed for each double infection each week.

Within 2 weeks after inoculation, symptoms of infection by the second virus were visible where either PCV or SCuM followed VBM. Where VBM was rubbed on plants infected with SCuM, however, no definite symptoms of infection by VBM developed. This was not surprising, since peppers that have been infected with SCuM for 2 months have almost stopped growing. SCuM, as expected (12), failed to produce symptoms in plants infected with the related PCV strain.

Titers of virus in the doubly infected plants were not similar to those found in singly infected ones (Fig. 1, D–F). When VBM was rubbed on plants infected with either SCuM or PCV, the challenge virus (VBM) failed to attain a titer as high as would be expected under conditions of a single infection. The effect was more pronounced with the VBM-SCuM combination than with VBM-PCV, both in terms of delay in reaching maximum concentration as well as in suppression of total titer. SCuM rubbed on plants infected with VBM failed to reach a high titer at any time during the 6 weeks of measurement. VBM titer was unaffected during the first month of the double infection but dropped rapidly after that time. Plants infected with PCV and then challenged by SCuM failed to develop SCuM in amounts sufficient for aphid recovery. This cross-protection action would be expected for these related viruses.

Discussion.—The differences found between the virus-activity curves of VBM and those of the cucumber mosaic viruses seem applicable in explaining the relative rates of field spread of these viruses. It is also worth noting that these results might be of some importance in the evaluating of chemicals that have antiviral properties. It seems likely that materials that suppress virus titer might be just as useful as are those that reduce susceptibility to infection.

The similarities in results with aphid and sap transmission of all 3 viruses suggest that aphids might be used to measure titers of active virus. Sylvester (14) recently has pointed out some of the advantages and disadvantages of this type of approach. It would appear that with a virus like VBM there may be definite possibilities of utilizing this technique. Since the work with VBM presented in this paper was done, some 23 additional trials involving green peach aphids transmitting VBM from pepper to pepper have been carried out. The amount of transmission (25–45 aphids used per trial) ranged from 63 to 82 per cent, with an average of 70 per cent and a standard deviation (S.D.) of 7.3 per cent. Twenty-one of the 23 samples fell within the range of 1 S.D. In the case of the cucumber mosaic strains, the efficiency of transmission is so low that it would require extremely large sample sizes to obtain truly comparative results.

The changes in titers of SCuM and PCV over a period of several weeks are similar to those reported for certain other nonpersistent aphid-borne viruses, alfalfa mosaic virus (8) and brassica nigra virus (14). The markedly different virus titer curve obtained with VBM, however, suggests that all viruses of this type do not behave similarly in their hosts. Certain differences in the physical properties of the viruses may account, at least in part, for this situation. Whereas all of the 5 viruses have similar dilution and thermal inactivation end points (10, 12, 14, 16), VBM appears to be more stable *in vitro* (10–15 days) than are the other 4 viruses (2–5 days). It would seem likely that the virus with the greater *in vitro* stability should also be the more stable one *in vivo* (2).

Work with tobacco mosaic virus indicates that the reduction in concentration of that virus that follows the initial maximum is the result of dilution due to plant growth (6, 13). A similar correlation between plant growth and reduction in active virus might be indicated in the case of the very mild PCV; however, a related strain, SCuM, has a titer curve similar to that of PCV, yet the effect of this virus on the plant is a near stoppage of growth within 3 weeks of inoculation. And in the case of VBM, although moderate stunting occurs, the titer of active virus remains con-

stant for at least 5 weeks after the initial maximum has been reached. It is apparent that titers of these viruses and growth of the host are not obviously correlated.

Results obtained with double infections indicated that these viruses behaved unlike certain others that have been tested. Most of the early studies (3, 7, 9) on multiple infections revealed a marked increase in the titer of 1 of the 2 viruses. Recent evidence (9), however, has shown that this phenomenon is dependent upon the combination of viruses used. Combination infections of VBM and either SCuM or PCV always resulted in reduced titers of total active virus. What may be a similar type of reaction has been reported with tobacco-etch virus and potato virus Y (1); in a mixed infection, etch virus replaces virus Y.

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CHLOROGENIC ACID CONTENT AND VERTICILLIUM WILT RESISTANCE OF POTATOES[1]

Shu-fung Lee[2] and Duane Le Tourneau

SUMMARY

The amount of chlorogenic acid present in roots and leaves of potato varieties with various degrees of resistance to Verticillium wilt was determined. Leaves contained more chlorogenic acid than did roots, whereas stems contained very little chlorogenic acid. Varieties resistant to Verticillium wilt contained more chlorogenic acid in the roots than did susceptible varieties. *In vitro* experiments showed that chlorogenic acid is metabolized by *Verticillium albo-atrum* Reinke & Berth. in liquid media and that chlorogenic acid can serve as a carbon source for the organism. Penicylinder assays on solid medium showed that chlorogenic acid was more inhibitory to the organism in neutral or alkaline media than in acid media. Even at high pH, large quantities (500–1000 ppm) of chlorogenic acid were necessary for inhibition of *V. albo-atrum*. The growth of *V. albo-atrum* was inhibited when the pH of liquid media was less than 3.4.

Verticillium wilt of potatoes, caused by the fungus *Verticillium albo-atrum* Reinke & Berth., has been of increasing importance in Idaho. Diseased plants produce small yields because of the early death of susceptible plants, and the fungus infects the tubers, making them useless for seed. In southeastern Idaho, on subirrigated sandy soils, the disease may curtail the yield by 50 per cent and is, in many cases, a limiting factor in potato production (7).

Many of the older potato varieties are not resistant to Verticillium wilt. The breeding of new resistant varieties is time consuming and is complicated by the fact that late-maturing varieties may escape the disease in the field without being resistant (7).

If it were possible to demonstrate a chemical basis for resistance of potatoes to Verticillium wilt, years of initial field screening might be partially replaced by appropriate chemical determinations. Such a chemical basis for resistance seemed possible when McLean et

[1] Accepted for publication December 20, 1957.
Published with the approval of the Director of the Idaho Agricultural Experiment Station as Research Paper No. 430. Portion of a thesis submitted by Miss Lee in partial fulfillment for the M. S. Degree.

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al (8) found by histochemical tests that tissues of Verticillium-wilt-resistant varieties of potatoes appeared to contain more chlorogenic acid than did tissues of susceptible varieties. This compound was previously implicated as responsible for resistance of potatoes to scab, caused by *Streptomyces scabies* (Thaxt) Waks. & Henrici (3, 10, 11).

The twofold purpose of this study was to investigate the distribution and concentration of chlorogenic acid in various parts of the potato plant and to determine the *in vitro* effects of chlorogenic acid and allied compounds on *V. albo-atrum*.

MATERIALS AND METHODS.—Seven varieties of potatoes, Populair, Great Scott, U.S.D.A. seedling 41956, Early Gem, Bliss Triumph, Kennebec, and Russet Burbank, were used. (Tubers were supplied by Dr. John McLean, Agricultural Research Service, U. S. Department of Agriculture, Branch Experiment Station, Aberdeen, Idaho.) Plants were grown in soil and in sand culture with Hoagland's solution in 2-gal. glazed crocks.

After a growth period in the greenhouse, the plants were harvested and separated into leaves, stems, and roots and washed gently to remove sand and dust. The tissues were then rapidly cut into small portions with a scissors, weighed, and blended in a Waring Blendor for 3 minutes in approximately 80–85 per cent ethanol (100 ml of 95 per cent ethanol were added for each 15 g of plant material). The blended material was boiled for 10 minutes, then the extract was filtered and the residue re-extracted 4 additional times with 80 per cent ethanol. The filtrates were combined, concentrated *in vacuo*, and made to a known volume containing about 10 per cent ethanol.

Chlorogenic acid was determined by paper chromatographic separation followed by ultraviolet spectrophotometric assay. A measured amount of sample was added with a micropipette to the starting line on a sheet of $7\frac{1}{2} \times 20$-in. Whatman No. 1 paper. After equilibration in the chromatography cabinet, the paper was irrigated with the organic phase of a system of butanol, acetic acid, and water (4/1/5, v/v/v) for about 18 hours. For some analyses, 20 per cent acetic acid also was used as the irrigant. This 1-phase mixture was not as effective as the 3-phase system in producing a well-defined band for chlorogenic acid in plant extracts; however, it worked quite well in qualitative studies of plant extracts and of culture medium. Following irrigation, the chromatograms were dried in a hood and the chlorogenic acid located by examination in ultraviolet light. The position of the blue fluorescent band of chlorogenic acid was verified by including known chlorogenic acid alongside the unknown. (One sample of authentic chlorogenic acid (hemihydrate) was obtained from Dr. Gestur Johnson and another from the Reheis Co.)

The fluorescent spot of chlorogenic acid was outlined lightly in pencil and then cut from the chromatogram. The spot was then cut into several small pieces and the acid eluted from the paper by 2 extractions with warm water (total of 10 ml). The eluates were filtered through a sintered glass crucible and the filtrates combined. A 3-ml aliquot of the filtrate was used for absorption measurements in a 1-cm light path at 325 mμ in a Beckman Model DU spectrophotometer. The same procedure was followed with known amounts of chlorogenic acid in the preparation of a standard curve. The amount of chlorogenic acid in the unknown was calculated from the known dilution factor.

Experiments to determine the *in vitro* effect of chlorogenic acid on *V. albo-atrum* were made with 2 synthetic media. The first of these was a sucrose-salts medium containing the following amounts of major salts and sugar per liter: $MgSO_4 \cdot 7H_2O$—0.5 g, KCl—0.5 g, KH_2PO_4—1.0 g, $NaNO_3$—3.0 g, and sucrose—30 g. The second medium, designated as glucose-asparagine medium, contained the following per liter: asparagine monohydrate—1.2 g, $MgSO_4 \cdot 7H_2O$—0.5 g, KH_2PO_4—1.5 g, and glucose—30 g. An aliquot of a minor element solution and of a ferric nitrate solution were added to both media so that the final concentrations per liter of media were as follows: Fe—0.2 mg, Cu—0.1 mg, Zn—0.1 mg, Mo—0.02 mg, and Mn—0.02 mg. Manganese, copper, and zinc were added as sulfates and molybdenum as sodium molybdate. After preparation, the stock medium was split into several portions and the test chemicals added. The pH was then adjusted by adding either dilute NaOH or HCl solutions. These media were then dispensed 25 ml per 125-ml Erlenmeyer flask; the flasks were plugged with cotton and autoclaved 15 minutes at a pressure of 15 lbs. In certain experiments, media were sterilized with a Seitz filter.

Inoculation, growth conditions, and mycelial harvests were as previously described (5). In some experiments, the culture medium was retained for analysis. It was collected in a beaker and boiled to inactivate enzymes. When necessary, the boiled filtrate was concentrated *in vacuo*.

Assays of chemicals also were made with penicylinders on solid medium. The sucrose-salts medium was solidified with 2 per cent agar and dispensed 25 ml per petri plate. The plate was seeded with 1.5 ml of a spore suspension prepared by flooding the surface of a 10- to 14-day-old culture with sterile distilled water. The same suspension was used to seed all plates in each experiment. After seeding, a sterile penicylinder was placed on the agar surface, and 0.1 ml of test material was added to each penicylinder. The zone of inhibition was measured after 48 or 72 hours.

Three isolates of *V. albo-atrum*, designated as 2, 4B, and 16B, were used for *in vitro* experiments. They differed in morphology in culture: Isolate 16B produced numerous microsclerotia, Isolate 4B usually produced a white mycelial mat but under certain conditions produced microsclerotia, and Isolate 2 appeared to be strictly a white mycelial type. (All isolates were isolated originally from diseased potato plants by Dr. James Guthrie of the Aberdeen Branch Station, Aber-

deen, Idaho.)

RESULTS.—*Chlorogenic acid content of potato plants.*—Preliminary paper-chromatographic experiments with extracts of potato roots and with a combined leaf and stem extract showed the presence of a material corresponding to chlorogenic acid (8). Chromatographic evidence included comparable R_f values in 3 irrigating systems: 1) butanol, acetic acid, and water; 2) phenol and water; and 3) the 1-phase solvent, 20 per cent acetic acid. When these irrigants were used, the spot corresponding to chlorogenic acid gave blue fluorescence under ultraviolet light, a blue color with Folin-Ciocalteu reagent, and a yellow to brown color when sprayed with 2 per cent $NaCO_3$ or when the chromatogram was sprayed lightly with water and exposed to ammonia fumes. In a further experiment, fluorescent spots that separated from root and leaf extracts and corresponded to chlorogenic acid were eluted from the chromatogram and their ultraviolet absorption spectra determined. The spectra coincided closely with that of known chlorogenic acid.

The amount of chlorogenic acid in roots, stems, and leaves of 7 varieties of potatoes was determined. The plants were grown in sand culture for 4 weeks, at which time the aerial portions of the plants were about 8–12 in. in height.

Leaves contained more chlorogenic acid than did roots (Table 1). It was not possible to obtain quantitative data of the chlorogenic acid content of the stems; only faint spots corresponding to chlorogenic acid were seen upon inspection of the chromatograms under ultraviolet light. Even though stem extracts were concentrated more than were those of roots or leaves and large quantities were analyzed, it was not possible to obtain a reading with the spectrophotometer. According to histochemical studies, chlorogenic acid is localized in the vascular system (8). With stem tissue, there undoubtedly is a preponderance of mechanical tissue as compared to vascular tissue. This probably would account for the low concentration in the stem.

TABLE 1—*The chlorogenic acid content of potato leaves and roots*

Variety	Resistance to Verticillium wilt[a]	Moisture content of leaves (%)	Percentage of chlorogenic acid[b]		
			Leaves		Roots
			Wet basis	Dry basis	Wet basis
Populair	Extremely resistant	92.0	0.17	2.1	0.08
41956	Very resistant	88.7	0.25	2.2	0.07
Great Scott	Resistant	90.7	0.12	1.3	0.11
Early Gem	Susceptible	91.7	0.17	2.0	0.01
Kennebec	Susceptible	90.9	0.14	1.6	0.05
Russet Burbank	Susceptible	86.1	0.18	1.3	0.01
Bliss Triumph	Very susceptible	90.6	0.08	0.9	0.03

[a]Resistance of potato varieties to *Verticillium albo-atrum* as determined by Dr. John McLean.

[b]Determined by paper chromatographic separation followed by ultraviolet spectrophotometric assay.

Results of chlorogenic acid determinations in the leaves show that the resistant seedling 41956 contains more chlorogenic acid than do the other varieties, whereas one of the most suspectible varieties, Bliss Triumph, contains less than do the other varieties. The values for the remaining 5 varieties fell between these extremes but not necessarily in the order of resistance to Verticillium wilt. The exact order depended on whether results were expressed on a dry- or wet-weight basis. This discrepancy was due to the relatively low moisture content of the Russet Burbank plants.

Results with roots show a correlation between chlorogenic acid content and Verticillium wilt resistance. The 3 resistant varieties, Populair, 41956, and Great Scott, all contained more chlorogenic acid than did those varieties listed as susceptible. These results agree with the qualitative histochemical tests of McLean et al (8).

In addition to chlorogenic acid, several other fluorescent materials previously encountered by McLean et al (8) were also located on chromatograms of the potato extracts. Leaf extracts of resistant 41956 and Populair and susceptible Russet Burbank and Bliss Triumph especially contained several fluorescent materials. Preliminary experiments were made to identify these materials by applying various spray reagents to the chromatogram. The fluorescent color under ultraviolet light and the color changes when the chromatogram is sprayed with an alkaline solution indicate that one material is an anthoxanthin and another is an anthocyanin.

In vitro effects of chlorogenic acid on V. albo-atrum.—1) *In media initially acid in reaction.*—Several experiments were made to determine the effects of chlorogenic acid on the growth of *V. albo-atrum*. In addition, caffeic acid and catechol also were compared in some of the tests. Caffeic acid contains an *o*-dihydroxy group and is a hydrolysis product of chlorogenic acid. Catechol was tested since it is the simplest *o*-dihydroxy phenol and because its effects on *V. albo-atrum* had been determined previously (5).

The 3 phenolic compounds were added to the sucrose-salts medium at a concentration of 10^{-3} M. This concentration was chosen since it has been shown that 10^{-3} M catechol is inhibitory to *V. albo-atrum* (5). The results of an experiment with Isolate 4B are summarized in Figure 1. Whereas catechol was quite inhibitory, chlorogenic acid at the same concentration had little if any effect on *V. albo-atrum*. Caffeic acid was intermediate in inhibiting the growth of the fungus. Somewhat similar results were obtained with the other isolates. Caffeic acid seemed to be less inhibitory to Isolate 2 than to the other isolates; it seemed quite similar to chlorogenic acid. The mycelial weight of Isolate 16B did not decrease during the later stages of growth. This is apparently due to the formation of microsclerotia.

In addition to the dry-weight changes, other quantitative and qualitative changes were noted. The growth

of the fungus altered the pH of the medium. After 30 days' growth, the medium had changed from the original pH 4.5 to pH 9. This change in pH was related to the growth of the fungus, as cultures inhibited by catechol did not show the rapid change in pH. The culture medium containing phenolic compounds changed from a light yellow to a dark brown during the course of the experiment. This color change was first noted about 3 days after seeding. The presence of phenolic compounds stimulated the production of microsclerotia, particularly when Isolate 16B was grown on media containing catechol.

It was found that concentrations of chlorogenic acid as high as 3×10^{-3} M (about 1200 ppm) were not inhibitory to *V. albo-atrum* in still culture in the sucrose-salts medium. Still cultures generally receive an inadequate or uneven oxygen supply. To overcome this limitation, seeded flasks were placed on the table of a gyratory shaker operating at about 85 oscillations per minute. The lag period was greatly shortened, and maximal dry weights of the fungus mats were obtained 4–6 days after seeding. As in the still culture, however, 10^{-3} M chlorogenic acid was not inhibitory to the fungus.

Since the sucrose-salts medium containing chlorogenic acid became progressively darker as the fungus grew, it seemed possible that chlorogenic acid was metabolized to colored quinone products. The application of a starch-KI test (9) showed a positive quinone test in the culture medium at the second and third harvests. In the later harvest, the culture medium no longer gave a positive quinone test; however, it is possible that the observed changes might also be due to the autooxidation of chlorogenic acid (2).

The use of a culture medium that would not become alkaline would obviate this difficulty. It was found that the pH of the medium did not become alkaline when *V. albo-atrum* was grown on the glucose-asparagine medium. In several experiments the maximum reading obtained never exceeded pH 6.3.

The effects of 10^{-3} M catechol, caffeic acid, and chlorogenic acid on *V. albo-atrum* in the glucose-asparagine medium were compared. Again, catechol was found to be more inhibitory than caffeic acid, which in turn was more inhibitory than chlorogenic acid. Chlorogenic acid may have inhibited slightly at the second and third harvests. As with the sucrose medium, the addition of a phenolic compound to the glucose-asparagine medium brought about increased production of microsclerotia.

Even though the glucose-asparagine medium did not become alkaline, it became progressively darker as the fungus grew. The culture filtrates collected at all the harvests were examined for the presence of chlorogenic acid by paper chromatography. Chlorogenic acid was found in the filtrates of the first and second harvests (5 days and 10 days after seeding). After the second harvest, chlorogenic acid was no longer detected. The filtrate of the second harvest contained a second fluorescent material; chromatographically, this material was indistinguishable from caffeic acid. This spot was not found in the other harvests nor was it found in the control medium of the second harvest. The transient presence of caffeic acid, a hydrolysis product of chlorogenic acid, and the disappearance of chlorogenic acid indicate that the fungus can hydrolyze chlorogenic acid and that it may metabolize the hydrolysis product.

In 1 experiment, chromatograms of culture filtrates of the first and second harvest showed the presence of small quantities of several fluorescent materials with R_f values less than that of chlorogenic acid. These materials appear very similar to those reported by Henze (1) and Weurman and Swain (17).

These results suggest that chlorogenic acid might serve as a carbon source for the fungus. To test this possibility, a mineral medium was prepared and the following added: 1) 1 per cent sucrose, 2) 1 per cent chlorogenic acid, or 3) 1 per cent chlorogenic + 1 per cent sucrose. The media were dispensed 10 ml per test tube, and duplicate tubes were seeded with 1 of 3 isolates. Visual observations showed that the fungus was not inhibited by the sucrose-chlorogenic acid combination and that chlorogenic acid alone could serve as a carbon source. The results obtained with Isolate 16B are shown in Figure 2. Media containing chlorogenic acid became dark brown in color. This discoloration showed up first directly beneath the surface mycelium.

2) *In media initially alkaline in reaction.*—Schaal and Johnson (11) reported maximum inhibition of *S. scabies* by chlorogenic acid when the reaction of the substrate was alkaline. The alkaline reaction allowed the chlorogenic acid to be autooxidized to the quinone. In the previous experiments, the initial pH of the media was 4.5. In the case of the sucrose medium, the pH became alkaline as the fungus grew and quinone, as indicated by the starch-KI test, was formed. Nevertheless, it seemed advisable to carry out experiments in which the initial pH was alkaline.

Initial studies were made with Seitz-filtered liquid media containing 10^{-3} M chlorogenic acid and adjusted to pH 4.5, 6.5, 7.0, or 8.5. After these flasks were seeded and incubated, visual observations indicated that chlorogenic acid at an original pH of 8.5 was more inhibitory than when the original pH was 4.5, 6.5, or 7.0.

The possible interaction of high initial pH and inhibition by chlorogenic acid was studied further. Instead of assays requiring large quantities of liquid media requiring Seitz filtration, penicylinder assays (on a solid medium) similar to those of Schaal and Johnson (11) were used. The solid medium was adjusted to the desired pH and then dispensed into the plates. Aqueous solutions of test materials were prepared and the pH adjusted just prior to use.

As in the liquid media, catechol was much more inhibitory than chlorogenic acid at an initial pH of 4.5. Growth on the plate was almost completely inhibited when 0.1 ml of 0.1 M catechol (1.1 mg) was added to the penicylinder. One-tenth ml of 0.1 M chlorogenic

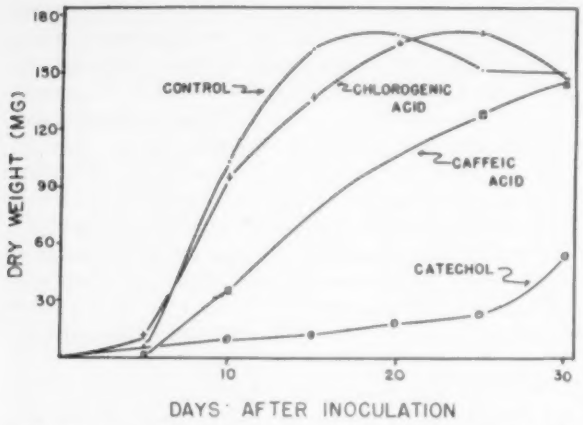

FIG. 1. The effect of 10^{-3}M catechol, caffeic acid, and chlorogenic acid on the growth of Isolate 4B of *Verticillium albo-atrum* in sucrose-salts liquid medium.

acid (3.5 mg) added to the penicylinder did not cause inhibition.

The effect of varying the pH of the media and the pH of the chlorogenic acid on the inhibitory effect of a constant amount of chlorogenic was determined. Table 2 is a summary of such an experiment with Isolate 16B of *V. albo-atrum*. Increasing the pH of the medium or of the chlorogenic acid increased the amount of inhibition.

It appears that on solid medium the quinone form of chlorogenic acid is more inhibitory than is the acid form. These results agree with those obtained by Schaal and Johnson (11) with *S. scabies;* however, the concentration of chlorogenic acid required to inhibit is rather high. Approximately 2–3 mg of chlorogenic acid per cylinder was required for inhibition. This amount in 25 ml of media represents about 100 ppm. When the concentration gradient within the plate is considered, it appears probable that inhibition at high pH probably occurs only in the range of 500–1000 ppm.

TABLE 2.—*Inhibition of Isolate 16B Verticillium albo-atrum by chlorogenic acid solutions adjusted to various pH values and added to penicylinders placed on seeded solid media[a] adjusted to various pH values*

pH of medium	pH of chlorogenic acid[b]	Inhibition zone in mm[c]
4.5	4.5	No inhibition
	6.0	14.5
	8.5	16.0
6.0	4.5	9.0
	6.0	14.0
	8.5	18.6
7.5	4.5	11.0
	6.0	13.2
	8.5	19.0
8.5	4.5	11.0
	6.0	13.2
	8.5	24.5

[a] Sucrose-salts medium.
[b] All treated plates received 0.1 ml of 0.1 M (3.5 mg) chlorogenic acid.
[c] After incubation for 72 hours.

3) *The effect of initial low pH on the growth of V. albo-atrum.*—Johnson and Schaal reported that chlorogenic acid added to unbuffered potato-dextrose agar lowered the pH sufficiently to retard the growth of *S. scabies* (3). They further suggested that chlorogenic acid in potatoes might lower the pH of the cells and thereby inhibit growth of the scab organism. McLean et al (8) made histochemical tests by applying an acid-base indicator to the freshly cut surface of various potato tissues. They found that vascular tissues of resistant varieties gave a deeper red when flooded with methyl red than did comparable tissues of susceptible varieties. This again suggested the possibility that pH of the cell contents may play a role in Verticillium wilt resistance.

In further experiments in liquid culture, sucrose and glucose-asparagine media were adjusted to pH 3.0, 3.2, 3.4, or 3.6 and seeded with Isolate 4B, 16B, or 2. An initial pH of 3.0 or 3.2 was completely inhibitory to all isolates. An initial pH of 3.4 inhibited growth for about 4 days after seeding, after which time the fungus began to grow. An initial pH of 3.6 did not retard growth. In addition, the interaction of low initial pH and the inhibitory effect of chlorogenic acid on *V. albo-atrum* was studied. The reaction of the sucrose medium was adjusted to pH 3.5 or 4.0, chlorogenic acid was added (10^{-3} M), and regular harvests were made. The initial pH of 3.5 caused a pronounced lag in growth rate, but the addition of chlorogenic acid did not enhance the effect.

DISCUSSION.—The results of the quantitative determinations support earlier histochemical findings that varieties of potatoes resistant to Verticillium wilt contain more chlorogenic acid than do susceptible varieties. This correlation between chlorogenic acid con-

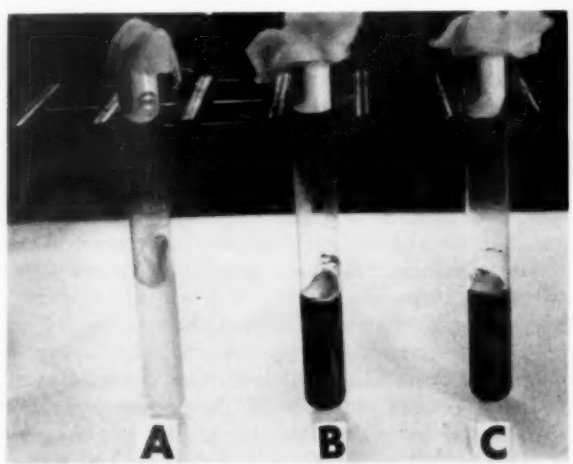

FIG. 2. Growth of Isolate 16B of *Verticillium albo-atrum* on salts media of pH 4.5 with various carbon sources. A) 1 per cent sucrose; B) 1 per cent chlorogenic acid; C) 1 per cent chlorogenic acid + 1 per cent sucrose. Growth period of 2 weeks.

tent and wilt resistance holds particularly in the roots of the varieties tested. The *in vitro* studies show that chlorogenic acid, at concentrations up to 1000 ppm, is not particularly inhibitory to the growth of *V. albo-atrum* in liquid media. In fact, chlorogenic acid appears to be metabolized and to serve as a carbon source for the fungus. These experiments were made in liquid media with mycelium as inoculum. It is possible that a study of the effects of chlorogenic acid on germinating spores or on germ tubes might yield different results. It is also possible that the concentration of chlorogenic acid in individual cells of potato plants might greatly exceed the concentrations used in these *in vitro* studies. Further studies to evaluate these possibilities are in progress.

Since the completion of this research, Valle (14) has reported that chlorogenic acid in potato leaves may be responsible for resistance to late blight caused by *Phytophthora infestans* d By. Similarly, Kuc et al (4) have reported that chlorogenic acid is inhibitory to the growth of *Helminthosporum carbonum* Ullstrup. It was found that inoculation of tuber tissue resulted in the production of chlorogenic acid. Thus, inoculation of the plant with a pathogen might also lead to production of more chlorogenic acid.

Both of these reports (4, 14) indicate that chlorogenic acid concentration of approximately 1000 ppm was necessary to inhibit the organism on solid media. In agreement with the work reported in this paper, caffeic acid was more inhibitory than chlorogenic acid to *H. carbonum* and *P. infestans*.

If chlorogenic acid is involved in wilt resistance, its effect may be due to its action in lowering the pH of the cells. It will be necessary to make further histochemical tests and pH determinations of cell sap and vascular fluid before this point can be clarified.

In an alkaline medium, chlorogenic acid is autooxidized to the quinone (2). Results of the study with solid medium demonstrate that chlorogenic acid is more inhibitory in alkaline than in acid media; however, the amount of chlorogenic acid required to inhibit *V. albo-atrum* even in alkaline medium is quite high. Before it can be accepted that the quinone form of chlorogenic acid is responsible for Verticillium wilt resistance, it will be necessary to determine the amount of chlorogenic acid in cells attacked by *V. albo-atrum* and also the pH changes occurring in cells invaded by the pathogen.

It also is possible that chlorogenic acid may be correlated with some other factor that controls resistance or that it may play only an indirect role. For example, Verticillium wilt resistance has been shown to be in some way associated with juvenility (7). This would suggest that disease resistance might be associated with high auxin content. Recently Waygood et al (16) have shown that certain phenols, such as catechol, competitively inhibit enzymatic oxidation of indoleacetic acid. It is possible that chlorogenic acid, in a similar manner, might be responsible for maintaining high levels of growth regulators in the plant.

Similarly, Kuc et al (4) found that cysteine added to solid medium enhanced the inhibitory effect of chlorogenic acid toward *H. carbonum*. It is possible that the concentrations of such material as cysteine might also vary within the plant and exert an effect on resistance. Such a mechanism would be consistent with the balance hypothesis of parasitism outlined by Lewis (6).

It has been shown that chlorogenic acid is responsible for enzymatic browning or discoloration of a variety of materials (2, 12, 13, 17). The blackening of the vascular system noted when potato plants are infected with *V. albo-atrum* may be due to enzymatic oxidation of chlorogenic acid by the fungus. Waggoner and Dimond (15) did not detect chlorogenic acid in potato stems and concluded that vascular discoloration was due to the liberation of substrates for polyphenol oxidase during pathogenesis. They further suggested that these substrates were liberated by the hydrolysis of conjugated phenols present in the plant. The results of the present investigation corroborate the presence of small quantities of chlorogenic acid in potato stems; however, if the chlorogenic acid is localized within the vascular system and if the supply is constantly renewed by translocation, it appears possible that there would be enough to account for vascular discoloration. In liquid medium, the fungus can bring about an intense darkening of chlorogenic acid, even at quite dilute concentrations.

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MATING TYPES AND OOSPORES OF PHYTOPHTHORA INFESTANS IN NATURE IN MEXICO[1]

M. E. Gallegly[2] and J. Galindo[3]

SUMMARY

Two mating types were found to occur among 95 Mexican isolates of *Phytophthora infestans* in a ratio approximating 1:1. Both mating types occurred among isolates taken from *Solanum* species growing in their native habitat and from commercial potato fields in Mexico. Oospores, resembling in all respects those formed in pure culture, were found in naturally infected potato leaves of the Katahdin variety growing in the Toluca Valley. Tracings of the antheridial and oogonial branches from 4 oospores produced by the pairing of 2 isolates of opposite mating type in culture revealed that the antheridial branches were produced by an isolate representing the only type found in the United States and that oogonial branches were produced by an isolate from Mexico that was of a mating type not found in North America. Additional tracings are necessary, however, to determine whether the mating types are controlled by compatibility factors, by factors that control sexual morphology, or by both.

The recent discovery by Smoot et al[4] that oospores of *Phytophthora infestans* (Mont.) d By. are formed in great abundance when isolates of opposite mating type are paired has answered an age-old question of sexuality in the species and helps explain why earlier workers failed to find oospores in abundance. The above workers pointed out that 2 mating types were found among 4 isolates from Mexico whereas 105 isolates from the United States, Canada, western Europe, South Africa, and the West Indies all reacted as 1 mating type. Three of the isolates from Mexico formed oospores in abundance when paired with any one of the group of 105 isolates or with a fourth isolate from Mexico. Oospores were not produced in abundance by pairing of isolates within each group.

Because both mating types were found among isolates from Mexico, it was desirable to study the mating reactions of a larger number of isolates from that country to determine whether there were compatible mating types in addition to the 2 previously described and to determine whether the sexual stage occurred in nature in Mexico where the 2 mating types were present. A preliminary report of these studies has been presented previously.[5]

Materials and methods.—The materials and facilities for this work were furnished by the Chapingo Laboratory of the Office of Special Studies of the Mexican Ministry of Agriculture. The Mexican cultures used in the mating studies were isolated by the personnel of the Chapingo Laboratory under the direction of Dr. John S. Niederhauser, who kindly placed them at the disposal of the authors. The cultures were isolated during the years 1952–1956 and had been maintained on dried-lima-bean agar. Mexican isolate 43M of Smoot et al[4] was used to represent one mating type and the United States isolates 63 and 256 were used to represent the other mating type. The isolates of the Mexican culture collection were then paired with 43M and with 63 or 256 on V-8 juice agar[4] in Petri dishes. As shown in Figure 1, the isolate of unknown mating reaction was placed in the center

[1] Accepted for publication December 20, 1957.

Based on cooperative investigations made with the office of Special Studies of the Mexican Ministry of Agriculture during the summer of 1956 when the senior author was in Mexico on a travel grant from The Rockefeller Foundation. Published with the approval of the Director of the West Virginia Agricultural Experiment Station as Scientific Paper No. 555. Journal Series Paper No. 83 of the Mexican Agricultural Program of the Rockefeller Foundation.

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[3] Ingeniero Agronomo, Oficina de Estudios Especiales, Secretaria de Agricultura y Ganaderia, Mexico.

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TABLE 1.—*Mating reaction of 95 Mexican isolates of Phytophthora infestans collected in different areas from 1952 through 1956*

Area of collection	No. of isolates collected in indicated year found to be of indicated mating type[a]											
	1952		1953		1954		1955		1956		Total	
	A^1	A^2	A^1	A^2	A^1	A^2	A^1	A^2	A^1	A^2	A^1	A^2
Popocatepetl	6	4							2	0	8	4
Toluca	19	10	3	1	5	4	1	4			28	19
Chapingo	5	6	3	0	6	13					14	19
Other	2	0			0	1					2	1
Total	32	20	6	1	11	18	1	4	2	0	52	43

[a] Isolates in group A^1 mated with Mexican isolate 43M whereas those in A^2 mated with United States isolate 256.

of the dish and the isolates of opposite mating reaction were placed on each side. Examination for oospore production was made on each side of the plate soon after juncture of mycelia from the 2 colonies.

In a search for oospores in nature, diseased leaf and stem tissue was collected from naturally infected plants of commercial-type potatoes and of several *Solanum* species growing in their native habitat; these were brought to the laboratory for microscopic examination. The diseased tissues were dissected, sectioned free-hand, or ground with a mortar and pestle before examination.

EXPERIMENTAL RESULTS.—*Mating reactions.*—The data in Table 1 show the mating reactions of 95 Mexican isolates of *P. infestans* collected in different areas from 1952 through 1956. Arbitrarily, the isolates that mated with Mexican isolate 43M were placed in group A^1 whereas those that mated with either of the United States isolates, 63 or 256, were placed in group A^2. Both mating types were obtained from the 3 areas in the vicinity of Mexico City. Of the 95 Mexican isolates tested, 55 per cent were of mating type A^1 and 45 per cent were of type A^2. In contrast, 18 isolates imported from the United States and Canada and maintained in Mexico all reacted the same as did isolates in group A^1.

Some isolates of the same mating type produced oospores in great abundance when paired with isolates of the opposite type whereas others produced them in relatively small numbers; however, all isolates produced oospores with one or the other of the standard isolates of opposite mating type. Of those tested none produced oospores with both. In some pairings, isolates of the same mating type tended to repel each other at the juncture of the 2 mycelia whereas those of opposite type did not (Fig. 1). In other pairings, this phenomenon was not clearly observed.

Oogonia with amphigynous antheridia typical of the sexual bodies formed when 2 compatible isolates were paired on V-8 juice agar are shown in Figure 2, A, B. Some of the Mexican isolates formed oospore-like bodies when grown alone; occasionally, perfectly formed sexual structures, oogonia with amphigynous antheridia, were seen in these cultures. All isolates producing these occasional oospores, however, gave a strong mating reaction when paired with isolates of opposite mating type.

In an effort to determine whether the 2 mating groups were morphological sexual groups or compatibility groups, attempts were made to trace the antheridial and oogonial hyphae from oospores to their original colonies. Although these attempts were made with cultures on water agar, the abundance of the hyphal branches made it difficult. Successful tracings were made from 4 oospores produced in 43M × 256 cultures. In these 4 cases, the antheridial branches were traced to the 256 colony and the oogonial branches to the 43M colony. To determine whether sexual groups, compatibility groups, or both exist, additional tracings from larger numbers of oospores produced in differently paired cultures will be necessary.

Oospores in nature.—Diseased leaves and stems of commercial varieties and of native *Solanum* species were collected at approximately weekly intervals from the Toluca, Chapingo, and Popocatepetl areas and brought to the Chapingo laboratory for examination. The first collection was made June 28, 1956, and the last collection on August 7. The first collections made in the early part of the blight season were of stems and leaves bearing few lesions whereas those made later in the season were of foliage with many lesions. The collection on August 7 consisted of leaves from the Katahdin variety growing in the Toluca Valley. Single leaves were placed in separate Petri dishes containing moistened filter paper and allowed to deteriorate for 6 days before examinations for oospores were made.

Nothing resembling oospores of *P. infestans* was

FIG. 1. Isolate 43M of *Phytophthora infestans* at top and isolate 256 of opposite mating type at the bottom, with an isolate of unknown mating type in the center. Oospores formed at the juncture of mycelia from the center and top colonies whereas mycelia from the center and bottom colonies repelled each other.

found until examinations were made of the material collected August 7. Oospores were found in moderate numbers in 1 of the 10 multiple-lesion leaves collected. These spores (Fig. 2, C-F) resembled in all respects those formed in agar culture from pairing of opposite mating types.

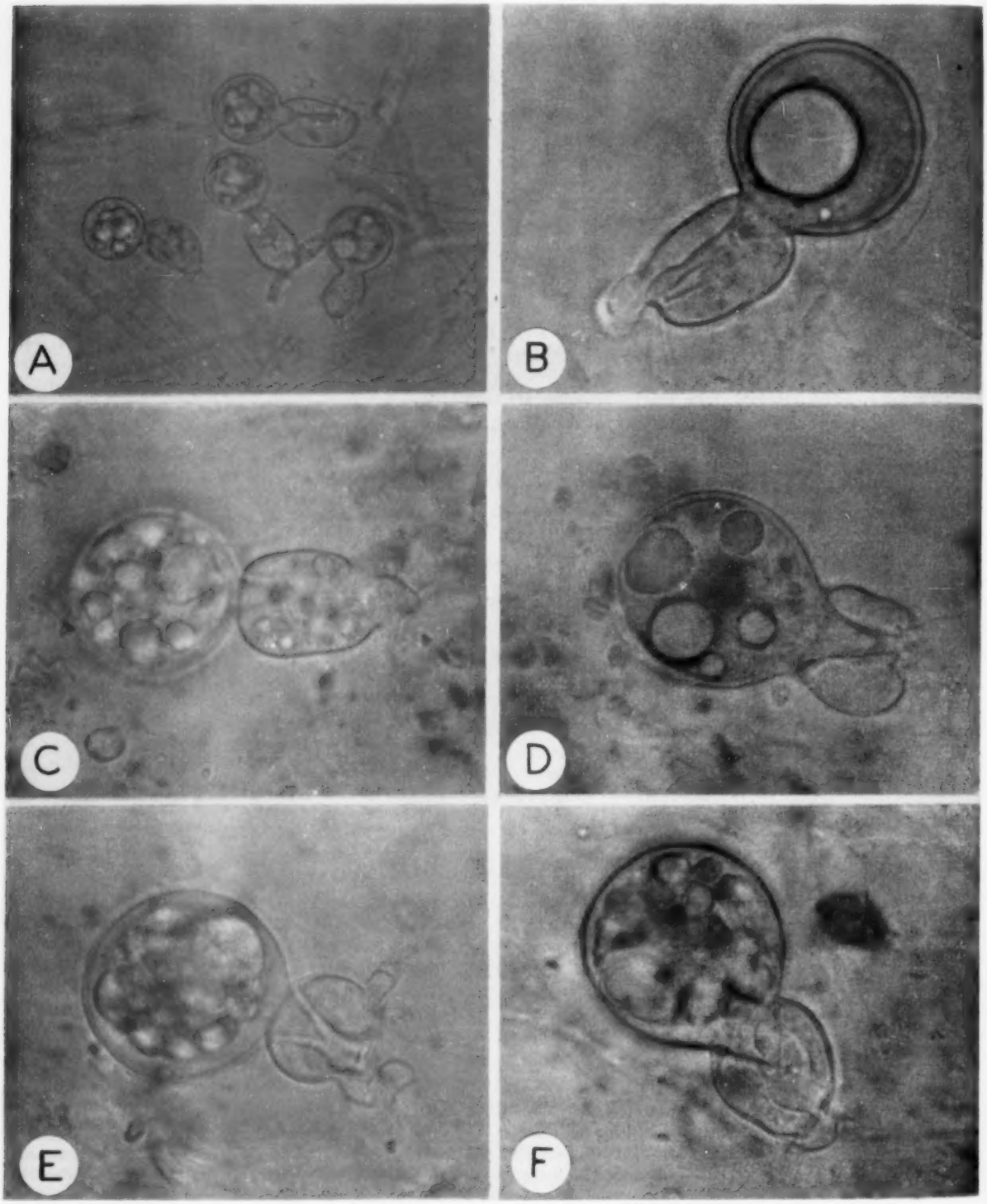

FIG. 2. A, B) Oogonia with amphigynous antheridia formed from pairing of compatible isolates of *Photophthora infestans* on V-8 juice agar (400× and 1200×, respectively). C, D, E, F) Oogonia with amphigynous antheridia found in naturally infected leaves of Katahdin plants growing in the Toluca valley (1200×).

DISCUSSION.—The results reported herein confirm the discovery[4] that 2 mating types exist in *P. infestans* and that oospores are formed in abundance when isolates of opposite types are paired in agar culture. In addition, the results show that both types are present in an approximate 1:1 ratio among isolates taken from nature in Mexico and that oospores resembling those formed in pure cultures occur in naturally infected potato leaves in a field where both mating types are present. Although it has not been proved by germination and plant inoculations that the oospores found in nature are those of *P. infestans*, the morphology of the spores found in the blight lesions leaves little room for doubt.

At present it is not known definitely whether the 2 mating types are compatibility types or whether they are morphological types. Tracings of the oogonial and antheridial hyphal branches from 4 oospores showed that the antheridial branches came from the United States isolate 256 and that the oogonial branches came from the Mexican isolate 43M. Although this might suggest that the 2 mating types are morphological sex types, the fact that many isolates, when grown alone on agar, occasionally formed a few oogonia with antheridia suggests that both genes for sex are present in these isolates and that they are highly self incompatible. Additional tracings from a larger number of oospores involving different pairings are needed before definite conclusions can be drawn. It is possible, of course, for both compatibility (1 or more gene pairs) and morphological factors to be involved in oospore formation. Regardless of which situation exists under natural conditions, the fungus is primarily heterothallic.

Since both mating types were found in approximately equal proportion in Mexico and since oospores were found in nature there, it is believed that the sexual stage plays an important part in physiologic race development and survival of *P. infestans* in that country. Niederhauser[6] has discussed the significance of these discoveries in relation to potato late blight in Mexico. Also, it seems logical to assume that the sexual stage will be found to play an important part in the late blight problem in other Central and South American countries where tuber-bearing *Solanum* species grow undisturbed in their native habitat.

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BLUEBERRY CANKER IN BRITISH COLUMBIA[1]

W. E. McKeen[2]

SUMMARY

Blueberries are becoming an increasingly important cultivated crop in British Columbia, but all the commercial varieties grown were bred in New Jersey.

Blueberry canker is responsible for wilting and death of numerous stems on older plants and in many instances for death of plants in young plantations. Canker is caused by *Godronia cassandrae*, the organism that causes storage rot of cranberries in Washington, Wisconsin, and eastern United States. Blueberry canker does not occur in any of the blueberry-producing areas of the United States, however, not even in the state of Washington. Environmental conditions would appear to be responsible in part for this anomaly. The cranberry and blueberry pathogens appear to be distinct races of *Godronia cassandrae* because the conidia from the cranberry isolate were nonpathogenic to blueberry and were straighter and slightly larger than the conidia from blueberry isolates.

Infection of blueberry occurs in the autumn, and cankers develop slowly during the winter. There is a range of resistance, Jersey being the most susceptible and rancocas the most resistant.

Blueberries, originally native North American wild fruits, are becoming an increasingly important cultivated crop in the Pitt Meadows area and in the peat bogs of Lulu Island, British Columbia. The first plantation was begun in 1923, and at the present time there are approximately 500 acres of blueberries. Not only has production doubled in the past 3 years, but it probably will increase another twofold in the next 5 years.

About 5 per cent of the acreage is planted on acid Ladner clay mineral soil, of which part is in sod and part is clean cultivated. The other plantations are on peat bogs, with acidity ranging from pH 3 to pH 4.5. Many of the plantations were started on the raw peat after the top 3 to 5 ft. of peat had been removed for commercial purposes. Deep parallel ditches approximately 100 ft. apart throughout the plantation serve to remove the water and lower the water table.

All the commercial varieties were bred in New Jersey, and in a great many instances only early-developed varieties such as rancocas and rubel have been planted. Harvest begins in early July and may continue through October if weather conditions are favorable, whereas

[1] Accepted for publication December 22, 1957.

[2] Associate Professor of Botany.

This study was conducted at the Plant Pathology Laboratory, University of British Columbia, Vancouver, B. C.

[6] Niederhauser, J. S. 1956. The blight, the blighter, and the blighted. Trans. N. Y. Acad. Sci. 19: 55-63.

in New Jersey picking is completed in July.

Because of their slow maturation in British Columbia, the plants are not sufficiently hardened and a great deal of winter tip injury frequently occurs. Also, because of abundant moisture in late September and October, fruit rot may be serious and shipment of fruit long distances is not feasible. The blueberry stunt virus disease (7), a serious disease in eastern North America, has not been reported in British Columbia probably because a suitable vector is absent. Some blight caused by *Pseudomonas syringae* V. Hall (10) has been observed on the Berkeley variety.

Blueberry canker is very serious; because of this and the lack of effective control measures, a study of this malady was undertaken.

Distribution and importance.—In 1931, Racicot and Hill (2) reported canker on a few plants in a depression in a small garden in Quebec. Racicot and Richardson (4) reported the same disease from British Columbia in 1943, and Creelman (5) stated in 1957 that canker was present in all plantations in Nova Scotia. In recent years, it frequently has been reported from British Columbia (6, 3).

This disease has not been reported from the United States; according to Scott,[3] it apparently is not present in the commercial plantation in New Jersey, Michigan, and Washington, where the varieties grown are identical with those grown in British Columbia.

In British Columbia, canker frequently kills 2 or 3 of the stems in each plant. Since no more than 2 or 3 stems are produced by young plants, either the young plant dies or it is unable to increase in size. Extreme difficulty is experienced in establishing a productive plantation. In 1 two-year-old plantation, 40 per cent of the plants were killed in 1955.

Old plants are seldom killed, but up to 25 per cent of the stems may be destroyed; as a consequence, yield is much less than normal.

Symptoms.—During the winter, incipient lesions appear (Fig. 10), usually centered about a leaf scar, on stems produced the previous year. These infection spots frequently occur at the ground level but many occur further up the stem. They enlarge rapidly in the spring, and in many instances coalesce with adjoining ones. They become black in color (Fig. 6), and pycnidial initials begin to appear during the latter part of March throughout the whole lesion (Fig. 7, 9). The pathogen develops very well in sunscald areas (Fig. 8).

[3] Letter from D. H. Scott, Principal Horticulturist, U. S. Department of Agriculture, Beltsville, Maryland.

Wilting of the infected stems (Fig. 1) begins during May and continues through the summer; usually all parts above the canker die. There is a marked variation in the varietal susceptibility, Jersey being the most susceptible and rubel and rancocas the most resistant.

Causal organism.—Canker is caused by *Godronia cassandrae* Peck. The apothecia (Fig. 5) only appear occasionally, at the base of 3-year-old stems. They are about 1 mm in diameter and have a narrow mouth that is almost closed when dry. The asci (Fig. 2) are cylindrical and measure 96.6–118.3 × 4.3–6.5μ; spores (Fig. 3) are filiform septate, nearly straight, and measure 51.6–68.8 × 1.6–2.2μ. Paraphyses are filiform and numerous. The pycnidia, which are abundant on all lesions, are subglobose to pyriform, rather thick-walled, more or less roughened, brownish, embedded at first but becoming erumpent when mature, and sessile or subsessile. Simple or irregularly chambered forms develop especially on artificial media. Spores (Fig. 4) are elliptic to fusiform, hyaline or very faintly yellowish in mass, pseudoseptate, and measure 8.4–17.0 × 1.2–1.9μ. This fungus is similar to *Godronia cassandrae* described on cranberry (8, 9), but the conidia of a cranberry isolate[4] from eastern United States were straighter and slightly larger than the conidia from blueberry isolates.

Godronia cassandrae was not found on any weed host. *Cassandra calyculata* L., a susceptible wild host (8), does not grow in southern British Columbia, the area in which blueberries are grown.

Infection studies.—It seemed obvious that at least some if not all infection occurred after June, because lesions occurred on parts of the stems that were formed during July and August. Infection in the autumn after the start of the wet season began seemed probable.

In the infection studies, isolates were used that were obtained from 1) single conidia, 2) single ascospores, and 3) a culture of *Godronia cassandrae* that was pathogenic on cranberry from eastern United States. The isolates made their maximum amount of growth at 20°C on potato-dextrose agar, and growth was inhibited at 30°. At 10° and 4°, radial growth was only 40 and 50 per cent, respectively, less than that at 20°.

The cultures obtained from the 3 sources were noticeably different when grown on cornmeal, oatmeal, potato-dextrose, or Lima bean agar. The cranberry isolate produced much aerial mycelium, and its color

[4] The culture was kindly supplied by Dr. H. F. Bergman, Senior Pathologist, U. S. Department of Agriculture, East Wareham, Mass.

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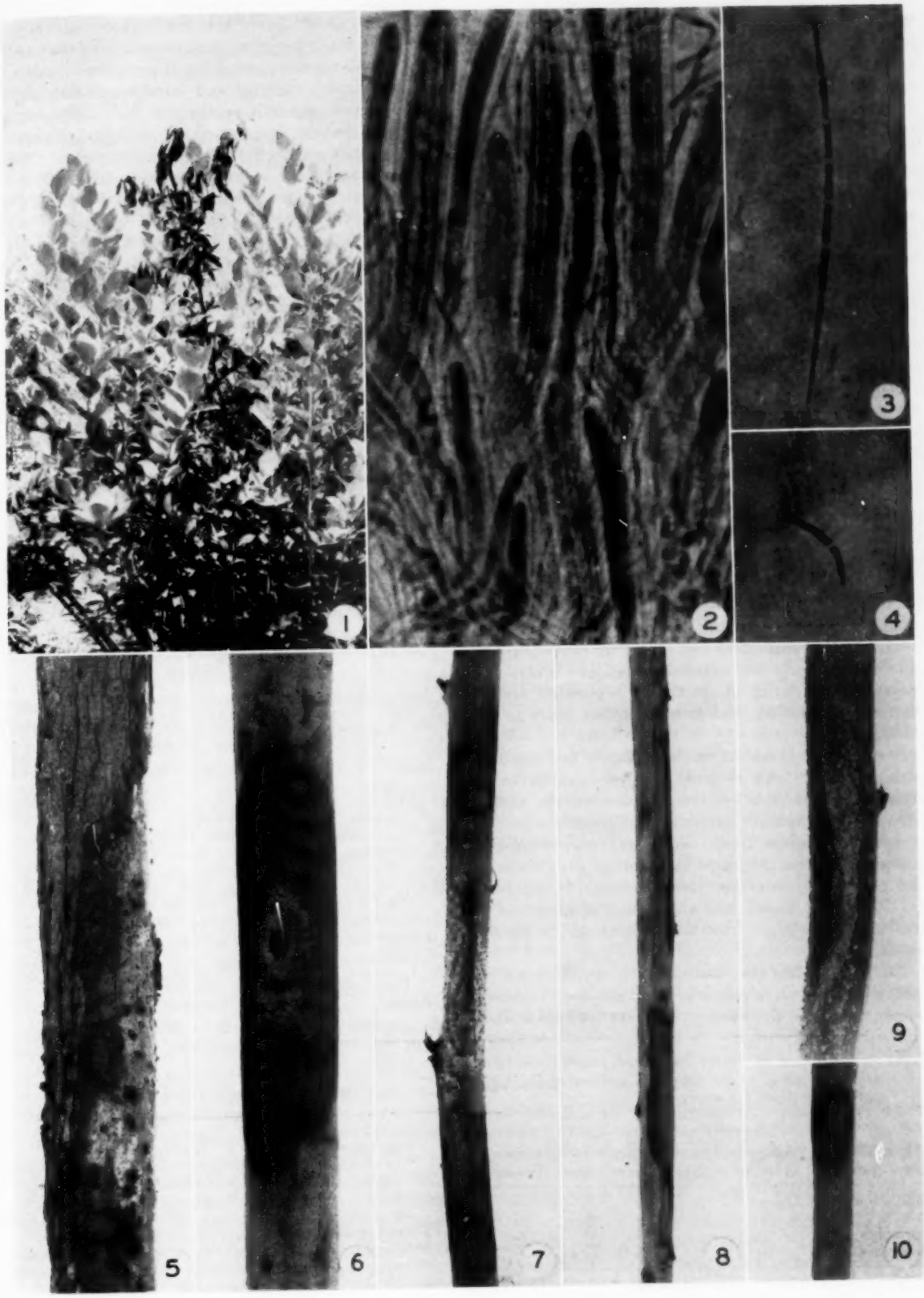
Fig. 1-10. Fig. 1. A dead stem, the result of a canker caused by *Godronia cassandrae*, in the center of a blueberry bush.—Fig. 2. Asci containing long septate spores along with paraphyses.—Fig. 3. A typical 8-celled ascus spore.—Fig. 4. A pseudoseptate conidium.—Fig. 5. A diseased cane with several apothecia.—Fig. 6. A typical black canker just prior to pycnidial formation. Note scar in center.—Fig. 7. A fruiting lesion.—Fig. 8. Pycnidia on the sun-scalded tissue.—Fig. 9. Pycnidial ooze on the tips of pycnidia.—Fig. 10. A young canker with a scar in its center.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varied from brown or buff to pink depending upon the medium upon which it was growing. It was the first isolate to produce pycnidia and produced them the most abundantly. The 2 isolates obtained from the ascospores produced no aerial mycelium but did produce black sclerotia on cornmeal and potato-dextrose agar. The 3 isolates obtained from the conidia grew approximately ⅓ as quickly as did those from the other sources. Except in the cornmeal agar, there was white or grey mycelium produced, and sclerotia as well as pycnidia were formed in all plates. The conidia from the cranberry isolate were straighter and slightly larger than those from the other 2 sources.

With spores from the 3 sources used in turn, 20 inoculations on the susceptible Jersey variety were made by each of 2 methods. In the first, the epidermis of the stem was punctured with an inoculating needle and then conidia that oozed from pycnidia were spread over these wounds. In the second, 20 inoculations were made by placing a drop of water containing hundreds of spores on one of the injured areas. The stems were inoculated at various places.

No infection resulted from the inoculations made in June and July with any of the isolates; infection occurred only on plants inoculated with conidia and ascospore isolates (i.e. the blueberry isolates) in November and December (Table 1).

Control.—No effective control measures have been worked out, but pruning and burning of diseased wood should help to alleviate this malady. Sprays with various fungicides applied in the spring by other pathologists failed to give any noticeable control. Sprays and dusts applied during August and September should destroy the inoculum and give protective cover to the stems.

Discussion.—Observations have shown that not only is this organism able to grow relatively rapidly in an artificial medium at a low temperature but also that it is capable of causing infection and canker development at low temperature. *Godronia cassandrae* also causes rot of cranberries at low storage temperatures. The reason why infection does not occur during June and July is not known, but either high temperature or degree of maturation of the host tissue could be the determining factor.

This study indicates that there is a difference in pathogenicity and morphology between the cranberry rot organism and the blueberry canker pathogen. It is interesting to note that pycnidia are produced abundantly on the blueberry host but only occasionally on cranberry fruit; however, the cranberry isolate was the most prolific in the production of pycnidia on artificial media. Further cultural and infection studies should be carried out to verify or disprove their differences.

Canker occurs on blueberries in British Columbia but does not occur in Washington State even though the same varieties are grown in both areas. The absence of disease in Washington could be due to unfavorable environmental conditions. The valley in which the blueberries are grown in Washington has a much higher summer temperature than does the area in which they are grown in British Columbia. The soil pH is equally low in these 2 areas, whereas it is much higher in New Jersey.

Conidia are obviously most abundant in the spring, but observations indicate that infection does not occur at this time. Fitzpatrick and MacSwan (6) apparently did not hold this belief, because they conducted spring spray tests. It seems probable that infection occurs during late summer and autumn rains. In this regard, blueberry canker may be similar to peach canker (1).

Fitzpatrick and MacSwan (6) state that no cankers were seen on the current-season growth, probably because of the extremely dry weather of 1951. Observations and results of the inoculation tests by the author show that cankers do not appear on the current-season growth.

Because of the scarcity of ascospores, they would appear to be of little importance.

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Table 1.—*Infection of Jersey blueberry plants inoculated at various times of the year with isolates of Godronia cassandrae from 3 sources*

Date of inoculation	Blueberry isolates [a]		Cranberry [a] isolate
	Ascospore isolate	Conidia isolate	
June 19	—	—	—
July 9	—	—	—
November 2	+	+	—
December 20	+	+	—

[a] — = No infection; + = infection.

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THE PERFECT STAGE OF HELMINTHOSPORIUM TURCICUM[1]

E. S. Luttrell[2]

SUMMARY

The perfect stage of *Helminthosporium turcicum* Pass. was produced in culture by mating compatible isolates. Mature ascocarps developed in 3 weeks when propylene-oxide-sterilized fragments of barley culm partially immersed in Sachs' agar plates were inoculated with blocks of mycelium from pairs of cultures of opposite mating type and held in an incubator at a constant temperature of 25°C. Conidial isolates from various hosts and locations in Georgia and single-ascospore isolates were approximately equally divided between the 2 mating types. The perfect stage is described as a new species of *Trichometasphaeria*. Perfect stages of *Helminthosporium* spp. have previously been described in the genera *Pyrenophora*, *Leptosphaeria*, and *Cochliobolus*. The diversity of perfect stages reported indicates the heterogeneity of the form genus *Helminthosporium*. Some correlations are apparent, however, between conidial characters and type of perfect stage.

Perfect stages of species of *Helminthosporium* occurring on grasses are distributed among 3 genera of Ascomycetes—*Pyrenophora*, *Leptosphaeria*, and *Cochliobolus*. In 1956 a fourth distinct ascocarpic type was discovered in matings of isolates of *H. turcicum* Pass. obtained from various locations in Georgia (16). Further study of this fungus has resulted in the development of a fairly satisfactory method of producing ascocarps in culture and a demonstration of heterothallism in this species. Morphological observations and a review of the taxonomic literature have led to the conclusion that the perfect stage is most closely related to species in the genus *Trichometasphaeria*.

MATERIALS AND METHODS.—Twenty-one single-conidium isolates were employed from leaf blight lesions on corn, sorghum, Sudan grass, and Johnson grass from various localities in south, middle, and north Georgia. Additional studies were made on 23 single-ascospore cultures isolated from ascocarps produced in culture. Isolations of conidia were made by brushing a sterile needle across the tips of the dense layers of conidiophores on the surface of lesions and streaking the dry spores across plates of plain water agar. After the spores had germinated, blocks of agar bearing a single conidium or hyphal tip, cut under low power of the microscope with a dissecting needle flattened at the tip, were inverted on thin plates of filtered 20 per cent V-8 Juice[3] agar so that further development could be followed by microscopic observation through the bottom of the dish. Single-ascospore isolations were made in the same manner except that a spore suspension was prepared by crushing mature ascocarps in drops of sterile water on sterile slides. Loopfuls of the suspension were streaked on water agar plates, and the ascospores were isolated at random. Cultures were maintained on V-8 Juice agar, which was a favorable medium for sporulation as well as for vegetative growth.

In attempts to produce ascocarps in culture, the following natural media were tried: fragments of barley straw, corn stalks, sorghum stalks, corn leaves, and corn and barley grains sterilized with propylene oxide and placed on the surface of, or immersed in, plates of water agar or Sachs' agar.[4] Inoculated plates were kept in incubators at temperatures of 10, 20, 25, and 30°C; in the laboratory at fluctuating temperatures; and out-of-doors during the fall months. The most consistent results were obtained with the following procedure:

Dry barley straw obtained at harvest time was soaked 2 hours in water. Two-inch sections of the culm surrounded by the leaf sheath were cut and placed in Petri dishes, which were stacked in a large glass jar to which was added 1 ml of propylene oxide per liter capacity of the jar. After 24 hours in the sealed jar, the dishes were removed. After an additional 24-hour period, Sachs' agar was poured into the dishes until the straw was half immersed and the plates were inoculated with pairs of isolates. Tiny blocks of one isolate were placed on one side of each straw; blocks of the second isolate were placed on the opposite side. The plates were held in an incubator at a uniform temperature of 25°C. Usually young ascocarps were apparent by the tenth day and mature ascospores after 21 days. Ascocarps developed only on the leaf sheath, not on the bare culm.

MORPHOLOGY AND TAXONOMY.—Isolates of *Helminthosporium turcicum* produced conidia abundantly on the exposed surface of the barley straw, and most isolates produced numerous tiny, black, sclerotium-like knots of hyphae on the straws above and below the agar surface. Sclerotium production varied among

[1] Accepted for publication Dec. 26, 1957.
Paper No. 324, Journal Series, Georgia Experiment Station.

[2] I am grateful to Mr. John A. Stevenson, Miss Alice L. Robert, and Miss Edith K. Cash for reviewing the manuscript and for their suggestions.

[3] A mixture of vegetable juices marketed by The Campbell Soup Company.

[4] Calcium nitrate—1.0 g, dipotassium hydrogen phosphate—0.25 g, magnesium sulfate—0.25 g, ferric chloride—trace, calcium carbonate—4.0 g, agar—20 g, water—1000 ml.

isolates. Sclerotia were also formed in plates of V-8 Juice agar. The ascocarps developed superficially on the leaf sheaths and could be distinquished from the more numerous sclerotia by their larger size and more regular contours. Although some ascocarps were formed on the exposed surface of the straws, the majority were on portions partially or completely immersed in the agar. Often they could be detected more readily by holding the plate up to the light and scanning, with a hand lens, the outlines of the straws through the bottom of the dish. Production of ascocarps was usually sparse and inconsistent, varying from straw to straw within the same plate and from plate to plate with the same isolates under apparently identical conditions. Development of adequate material for morphological or genetic study, therefore, was uncertain and required numerous repetitions of tests.

The ascocarp apparently was an ascostroma. Mature ascocarps (Fig. 4) were black and ellipsoidal to globose. They measured 359–721 μ in height and 345–497 μ in diameter. The outer layers were composed of a pseudoparenchyma of small cells with thick, dark-brown walls. Over the upper third of the ascocarp and around the ostiole the surface cells produced numerous short, stiff, brown, spine-like hairs (Fig. 3, 4). The inner wall layer was composed of small hyaline cells, partially exposed at the apex of the ascocarp by weak development of the darker outer layers at that point. The ostiole was formed as a result of the disruption of the thin-walled cells in that region. There was usually no evidence of a beak although, under moist conditions in culture, a mass of hyaline cells was often pushed out at the apex, and some of these cells produced short hyphae (Fig. 4).

The locule was occupied by a mass of hyaline, filamentous pseudoparaphyses extending downward from the inner wall cells across the upper part of the ascocarp (Fig. 1). Asci grew upward among the pseudoparaphyses from the base of the locule. The asci (Fig. 1) were cylindrical or clavate-cylindrical, with a short stipe, and measured 176–249 × 24–31 μ (mean 201.8 × 26.6 μ). The walls were thick, especially in young asci. The asci were typical of the bitunicate type, with a thin outer wall that split at the apex upon spore discharge, permitting expansion of the thick, extensible inner wall. Some asci were abnormal, being lobed in a Y-shaped fashion at the tips. No asci were found with a full complement of 8 ascospores; the number of mature spores varied from 1 to 6; 2 or 4 were most common.

The ascospores (Fig. 1, 2) typically were hyaline at full maturity. In over-mature ascocarps they sometimes became brown, but this coloration was accompanied by collapse of the asci, browning of the pseudoparaphyses, and other signs of degeneration in the centrum. They were fusoid, straight or slightly curved, typically 3-septate, and constricted at the septa. Spores with as many as 6 transverse septa were not uncommon. The larger, multiseptate spores usually developed in asci with only 1 or 2 spores. Ascospores measured 42–78 × 13–17 μ (mean 61.7 × 14.7 μ). The free ascospores often appeared to be surrounded by a long, thin, tubular, mucuous sheath, scarcely visible along the sides of the spore but extending beyond either end by as much as the length of the spore. This sheath usually was not detectable around spores within the ascus and frequently was not apparent around free spores. However, it occasionally persisted around spores that had germinated on agar.

The bitunicate asci, apparently ascostromatic ascocarps, and pseudoparaphysate centrum place this fungus in the order Pleosporales of the Loculoascomycetes (15). Because of the hyaline ascospores it would be placed in the genus *Metasphaeria*, as formerly recognized. However, in a recent taxonomic study Müller (20) reduced *Metasphaeria* to synonomy with *Leptosphaeria* since he considered spore color an unreliable generic character. As constituted by Müller, the genus *Leptosphaeria* includes a number of distinctive groups of species. Holm (12) recognized several of these groups as genera, among them the genus *Trichometasphaeria* (22, 23). Relationship with species in the genus *Trichometasphaeria* as delimited by Holm is indicated for the perfect stage of *Helminthosporium turcicum* by the following combination of characteristics: ascocarps lacking a beak and covered in the apical region with stiff hairs, clavate-cylindrical asci containing a variable number of ascospores, and hyaline, fusoid, phragmosporous ascospores provided with a mucous sheath. In having a long tubular mucous sheath around the ascospores it is similar to *L. artemisiae* (Fckl.) Auersw. (20); in the structure of the ascocarp it resembles *T. culmifida* (Karst.) Holm (12). It differs from other species in *Trichometasphaeria*, and from almost all species in *Leptosphaeria*, in its much larger ascospores as well as in its conidial stage. Consequently, the fungus is described as a new species of *Trichometasphaeria*, as follows:

Trichometasphaeria turcica sp. nov.

Ascomatibus superficialibus, sparsis, nigris, ellipsoideis vel globosis, 359–721 μ altis, 345–497 μ latis, poro praeditis, non rostratis, ad apicem pilis simplicibus, rigidis, brevis, brunneis, continuis vestitis, contextu coriaceo-carbonaceo, pseudoparenchymatico; ascis bitunicatis, cylindraceoclavatis, brevi pedicellatis, parieto crasso, 1–6-sporis (media 3.3-sporis), 176–249 × 24–31 μ (media 201.8 × 26.6 μ), pseudoparaphysibus numerosis, filiformibus; ascosporis hyalinis, fusoideis, rectis vel leniter curvulis, 2–6-, typice 3-septatis, ad septa constrictis, strata mucoso, tubuloso, tenui obtectis, 42–78 × 13–17 μ (media 61.7 × 14.7 μ).

Status conidicus: *Helminthosporium turcicum* Pass.

Hab. cultura in stramine Hordei vulgaris.

Specimens (Luttrell No. 6001, type) have been deposited in the National Fungus Collections, Beltsville, Maryland, and the Herbarium of the Royal Botanic Garden, Kew. Compatible isolates have been placed in the American Type Culture Collection and the Centraalbureau voor Schimmelcultures.

HETEROTHALLISM.—Ascocarps were first obtained in a mating of isolates No. 1170 (Sudan grass, Blairsville) and No. 1171 (corn, Blairsville). As a means of reference, isolate No. 1171 was designated a "plus" strain, isolate No. 1170 a "minus" strain. From the ascocarps produced in this cross, 12 random single-ascospore isolations were made. After these isolates had been tested in various combinations, 2 isolates of opposite sex reaction that produced ascocarps abundantly, No. 1198–9 (plus) and No. 1198–6 (minus), were selected for use, along with the original conidial isolates, No. 1171 and No. 1170, as testor strains in determining the sex reaction of the other isolates.

Of 21 single-conidium isolates from field material, 9 were plus strains, 9 were minus strains, and 3 failed to produce ascocarps in any matings with testor strains. Of 23 single-ascospore isolates, 11 separated into plus and 12 into minus strains. Ascocarps were produced only in pairings of strains of opposite sex reaction. None were produced by any single-conidium or single-ascospore isolate alone or by combinations of any 2 plus strains or any 2 minus strains. Although the

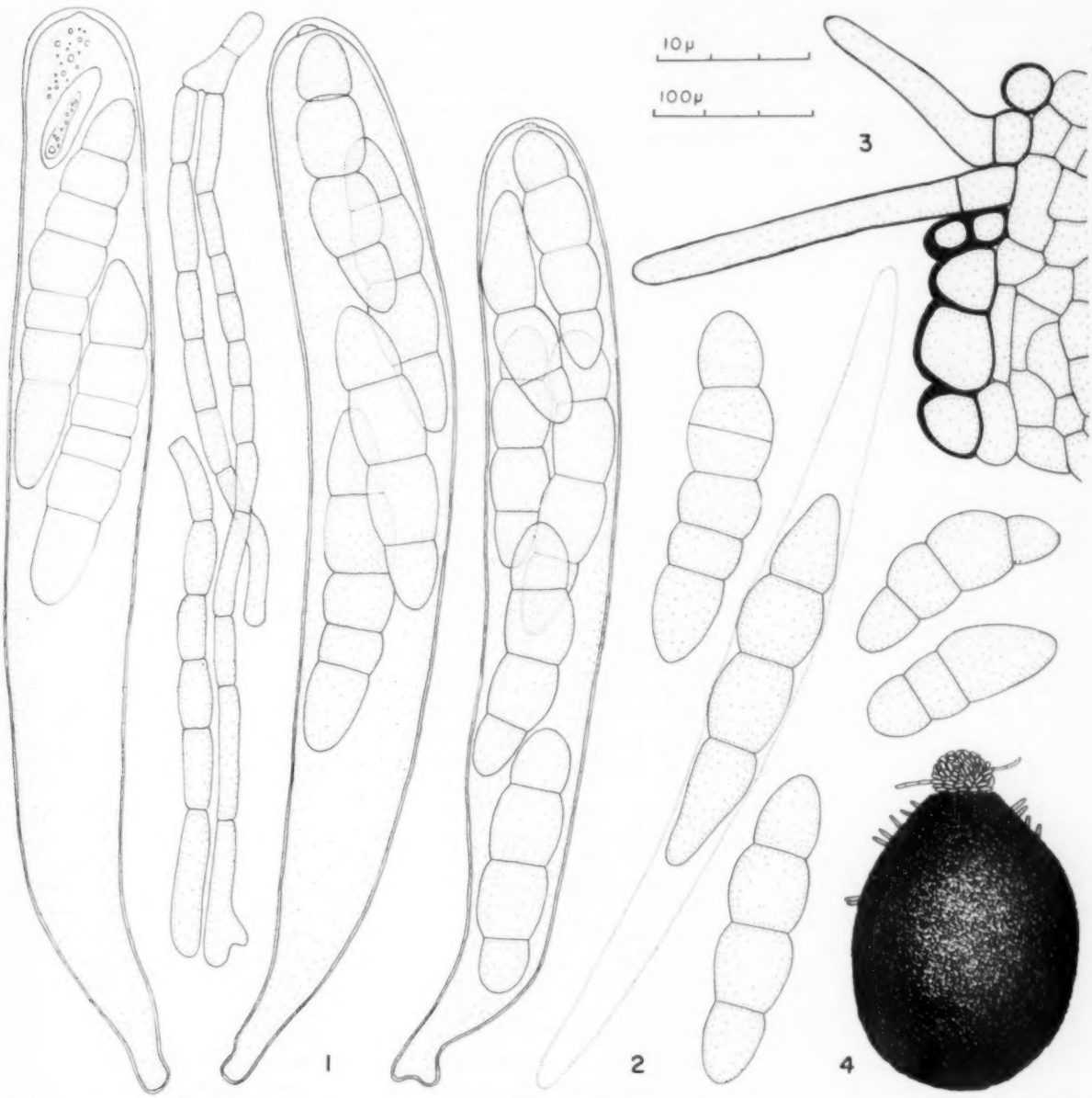

FIG. 1–4. *Trichometasphaeria turcica*.—Fig. 1. Pseudoparaphyses and 2-, 4-, and 6-spored asci.—Fig. 2. Ascospores, one showing tubular mucous sheath.—Fig. 3. Section of peripheral layers of ascocarp showing hairs produced as outgrowths from thick-walled superficial cells.—Fig. 4. Mature ascocarp with sparse development of hairs over upper part and mass of hyaline cells protruding through ostiolar region.—Fig. 1–3 to upper scale, fig. 4 to lower scale.

physical basis of sex in *Trichometasphaeria turcica* has not been determined, these data are sufficient to demonstrate that this fungus is heterothallic, i.e. strains of opposite sex reaction type exist and ascocarps are produced in matings of compatible strains. The approximately 1:1 segregation ratio for sex reaction type among the limited number of single-ascospore isolates tested suggests that sex reaction is determined by a single pair of factors. Among the 23 single-ascospore isolates, as indicated by the relative numbers of ascocarps produced in matings with the testor strains, 7 of the plus strains showed a strong and 4 a weak sex reaction, and 6 of the minus strains showed a strong and 6 a weak sex reaction. Matings between weak plus and weak minus strains usually failed to produce ascocarps. This further suggests that one or more pairs of secondary factors may have some effect in determining the intensity of the sex reaction. However, a proper genetic study will be necessary to demonstrate these points satisfactorily.

Determinations of sex reaction in isolates from field collections revealed no geographic or host limitation of the two sexual strains. Sex reaction of isolates from different hosts were as follows: corn—6 plus, 1 minus, 2 undetermined; Sudan grass—1 plus, 4 minus; Johnson grass—2 plus, 2 minus, 1 undetermined; sorghum —2 minus. Both plus and minus strains were isolated from field material collected at Tifton, in south Georgia; Experiment, in middle Georgia; and Blairsville, in north Georgia. In fact, isolates No. 1171 (plus) and No. 1170 (minus) were obtained from corn and Sudan grass plants growing close together in the same field at Blairsville. It should, therefore, be possible to find the perfect stage in nature if an intensive search is made under proper conditions.

Discussion.—The variety of perfect stages reported for species of *Helminthosporium* on grasses re-emphasizes the fact that *Helminthosporium* must be considered a form genus composed of species belonging to diverse natural groups. However, groupings of species within this genus on the basis of conidial characters may to some extent indicate natural relationships since, in some cases, these conidial characters are correlated with the type of perfect stage produced.

In the subgenus Cylindro-Helminthosporium, which was segregated as the genus *Drechslera* by Ito and Kuribayashi (13), the perfect stages of all species in which the life cycle is known belong in the genus *Pyrenophora*. These include *P. avenae* Ito & Kurib. (13) for *H. avenae* Eidam; *P. bromi* (Died.) Drechs (8) for *H. bromi* Died.; *P. graminea* Ito & Kurib. (13) for *H. gramineum* Rab.; *P. japonica* Ito & Kurib. (13) for an unnamed species similar to *H. teres* Sacc.; *P. lolii* Dovaston (7) for a species considered to be *H. siccans* Drechs., though its identity remains in some doubt; *P. phaeocomes* (Rebent.) Fr. for an unnamed Cylindro-Helminthosporium species (11); *P. polytricha* Da Camara (4) for *H. olisipponense* Da Camara; *P. teres* (Died.) Drechs. (8) for *H. teres*; *P. tritici-repentis* (Died.) Drechs. (8) for *H. tritici-repentis* Died.; *P. tritici-vulgaris* Dickson (6) for *H. tritici-vulgaris* Nisik.; and *P. secalis* Whitehead & Dickson (39) for *H. tuberosum* Atk. (fide Shoemaker; 30). In addition, *H. penicillosum* Speg. was found associated with immature ascocarps of *P. chaetomioides* Speg. (28).

The name *Pyrenophora* is of doubtful validity. Although Drechsler (10) favored its use for the perfect stages of these *Helminthosporium* species, Wehmeyer (37) concluded that, if *Pyrenophora* is to be maintained, it must be used for a form genus in the Fungi Imperfecti. Wehmeyer (36) placed a number of these *Pyrenophora* spp. in the genus *Pleospora* as synonyms of *Pleospora trichostoma* (Fr.) Ces. & de Not., which as thus constituted included strains producing conidial stages in *Helminthosporium* as well as strains lacking such stages. He recognized *Pleospora trichostoma* and related species as a distinctive subgroup that might be given generic rank under a new name, but retained this group in *Pleospora* pending a determination of its taxonomic limits. Wehmeyer's (36) treatment of these species emphasizes the fact that, for the most part, there are only slight differences among the various perfect stages of Cylindro-Helminthosporium species. It is also true that many of these species are difficult to distinguish in their conidial stages. A careful comparison of these fungi, considering the morphology of both the perfect and imperfect stages as well as differences in pathogenicity, will be necessary to resolve these taxonomic problems. In the meantime these species, which cause different diseases of various cereals and grasses, may be provisionally referred to the familiar combinations in *Pyrenophora* (21).

The *Pyrenophora* perfect stages of Cylindro-Helminthosporium species are typical members of the Loculoascomycetes, as is indicated by Wehmeyer's (38) developmental study of the closely related *P. trichostoma* (Fr.) Fckl. The ascocarps are commonly found in the field, where they have a long period of development. The stromatic initials develop in summer, on such crop refuse as stubble and straw, and the ascocarps mature during the fall or following spring. The few species brought to maturity in culture required long periods of development at low temperatures. *P. lolii* (7) produced mature ascocarps in cultures on oatmeal agar kept out-of-doors from September to February in Scotland. Mature ascocarps of *P. bromi* developed in cultures on potato-dextrose agar grown at room temperatures for 3–4 weeks and then kept at 5°C for 3 months (2). No ascocarps matured in cultures kept above 12° C. *P. trichostoma* produced mature ascocarps in cultures on Leonian's agar in a cold chamber at 5°C for 20 weeks after 18 days at room temperatures (31). All 3 of these species proved homothallic. By exposing cultures to alternating low and high temperatures or by holding them for shorter periods at temperatures as low as −20°C, Wehmeyer (38) was unable to reduce the time of 4 months required at 2–5°C for the development of mature ascocarps of *P. trichostoma*. Rathschlag (27) once produced ascocarps, which he

described as *Pleospora avenae* Schaffnit & Rathschlag, in a culture of *H. avenae* grown 10 days at room temperatures, exposed 6 days to −11°C, and returned for 11 days to room temperatures. He was unable to duplicate these results, however, and his illustrations of asci and ascospores indicate that he had been dealing with ascocarps of *Pleospora herbarum* (Pers. ex Fr.) Rab. contaminating his culture of *H. avenae*. *Pleospora avenae* Schaffnit & Rathschlag is clearly distinct from *Pyrenophora avenae* Ito & Kurib. and is probably synonymous with *Pleospora herbarum*.

The subgenus Euhelminthosporium is more heterogeneous than Cylindro-Helminthosporium. The species differ in conidial morphology and, correspondingly, in type of perfect stage produced. In typical representatives of Euhelminthosporium the perfect stages known fall in *Cochliobolus*, a genus established by Drechsler (10) for species formerly included in *Ophiobolus* but differing in having larger asci, filiform ascospores coiled in a helix, and *Helminthosporium* imperfect stages. The following species have been described: *C. boutelouae* Sprague (32), which possibly represents the perfect stage of *H. buchlöes* (Ell. & Ev.) Lefebvre & A. G. Johnson, although no connection with any conidial stage was established; *C. heterostrophus* (Drechs.) Drechs. (10) for *H. maydis* Nisik. & Miyaki; *C. kusanoi* (Nisik.) Drechs. ex Dastur (25, 5) for *H. kusanoi* Nisik.; *C. miyabeanus* (Ito & Kurib.) Drechs. ex Dastur (13, 5) for *H. oryzae* Breda de Haan; *C. nodulosus* Luttrell (17) for *H. nodulosum* Berk. & Curt. ex Sacc.; *C. sativus* (Ito & Kurib.) Drechs. ex Dastur (13, 5) for *H. sorokinianum* Sacc. ex Sorokin; *C. setariae* (Ito & Kurib.) Drechs. ex Dastur (13, 5) for *H. setariae* Saw.; *C. sporoboli* E. Castell. (3) for an undetermined species of Euhelminthosporium on leaves of *Sporobolus*; *C. stenospilus* (Carpenter) T. Matsu. & Yamamoto (18, 19) for *H. stenospilum* Drechs.; and *C. tritici* Dastur (5) for an unnamed species of Euhelminthosporium developed on "black point" wheat kernels on moist filter paper.

Shoemaker's (29) developmental study of *C. sativus* indicated that the ascocarp is initiated by a free ascogonium and should be considered a perithecium. Further, there is no evidence that the ascus in any species of *Cochliobolus* is bitunicate. *Cochliobolus*, therefore, is distantly related to other *Helminthosporium* perfect stages. It apparently belongs among the true Pyrenomycetes in the subclass Euascomycetes, although its proper taxonomic position here will be difficult to determine.

Perithecia of *C. boutelouae* (32), *C. kusanoi* (25), *C. miyabeanus* (6), and *C. sporoboli* (3) have been found in the field. Perithecia of *C. kusanoi* (25) and *C. miyabeanus* (13) have also been produced in culture. Perithecia of the remaining species are known only from culture. They have a short period of development and do not require exposure to low temperatures. Drechsler (9) obtained perithecia of *C. heterostrophus* on naturally infected corn leaves placed in a moist chamber. Ito and Kuribayashi (13) had amazing success in inducing perithecium formation in *C. heterostrophus*, *C. miyabeanus*, *C. sativus*, and *C. setariae* by the following method:

Naturally infected grain or leaf fragments were sterilized with mercuric chloride, washed, placed on rice culm decoction agar in Petri dishes, and incubated at 25°C. Mature perithecia of all species developed within 4–5 weeks. Corn meal agar was less satisfactory. (Perithecia of *C. miyabeanus* and *C. heterostrophus* were also developed on leaf fragments on moist filter paper or quartz sand.) Although perithecia were produced in cultures at laboratory temperatures, a constant temperature of 25°C was more favorable.

Nisikado (25) reported the production of perithecia by a single-conidium isolate of *C. kusanoi* on rice agar in 7–8 weeks at 11–15°C. Matsumoto (19) found that perithecia of *C. stenospilus* were produced within 5 weeks on pieces of infected sugar cane leaf that were surface-sterilized and placed on plates of potato-dextrose agar. Single-conidium isolates produced perithecia in 4 weeks at 25°C on sterilized sugar cane leaf and on onion, apricot, sugar cane leaf, and malt agars. Perithecia of *C. tritici* developed when a single conidium isolate was used to inoculate heads of wheat plants and the resulting "black point" kernels were placed on agar plates (5). This isolate did not produce perithecia when surface-sterilized wheat and rice grains were inoculated with it and incubated in moist chambers.

Unfortunately, no attempts were made to use single-ascospore cultures in any of the investigations on *Cochliobolus* spp. that have been mentioned. Studies employing single-ascospore isolates have been made recently on 3 species, *C. sativus* (29, 34), *C. heterostrophus* (24), and *C. nodulosus* (17). They proved to be heterothallic. In each of these studies perithecia were obtained by the laborious process of systematically mating isolates from field collections until compatible strains were discovered. The two sexual strains of these species might be expected to occur together naturally, as indicated by the fact that Nelson (24) isolated both sexual strains of *C. heterostrophus* from corn plants in a limited area in Georgia, and even from a single lesion, and Luttrell (17) obtained compatible isolates of *C. nodulosus* from a single location. Nevertheless, it is difficult to reconcile the fact of heterothallism with the apparent ease with which Drechsler (9), Nisikado (25), Ito and Kuribayashi (13), and Matsumoto (19) obtained perithecia by the incubation of naturally infected plant material or in single-conidium cultures.

The methods used in obtaining *Cochliobolus* perithecia in matings of compatible sexual strains have been essentially similar. All involved inoculation of the two strains onto sterilized plant parts placed on plates of Sachs' agar. Working with *C. sativus*, Tinline (34) placed barley grains, surface-sterilized with mercuric chloride, on the surface of Sachs' agar plates and inoculated them with a suspension of conidia of the

isolates being tested. The plates were held in an incubator at 25°C for 1 week, then placed at 20°C for 3–4 weeks. Shoemaker (29) found that corn grains similarly treated were a more favorable medium for this species. Nelson (24) produced perithecia by placing autoclaved squares of corn leaf on the agar and inoculating the squares on opposite sides with blocks of mycelium from cultures of *C. heterostrophus*. Perithecia matured in 3–4 weeks in plates held at a constant temperature of 24°C. Perithecia of *C. nodulosus* developed on fragments of barley straw sterilized with propylene oxide and partially immersed in Sachs' agar when they were inoculated with conidial suspensions or mycelial blocks and incubated at 25°C for 1 week and thereafter at 20°C for 3–4 weeks, or incubated at a constant temperature of 25°C (17).

Helminthosporium sigmoideum Cav. differs strikingly from other species of Euhelminthosporium in its acute fusoid conidia, which are borne on short stipes on the conidiophores, and in its peculiar sclerotial stage (*Sclerotium oryzae* Catt.). Because of these distinctive characteristics, Subramanian (33) established a separate genus for this species and created the combination *Vakrabeeja sigmoidea* (Cav.) Subramanian. *H. sigmoideum* likewise differs in its perfect stage, which was demonstrated to be *Leptosphaeria salvinii* Cav. (35). *L. salvinii* appears to be a typical *Leptosphaeria* with medium-sized, brown, 3-septate ascospores. However, Putterill (26) suggested that this species might be misplaced in *Leptosphaeria*. In view of Tullis' (35) description of the ascus wall as very thin and deliquescent at maturity of the ascospores, a study of the morphology and development of the ascocarp would be desirable to establish its taxonomic position. Mature ascocarps of *L. salvinii* were found on rice stubble in the field from October through February. They also developed within a short period on the dead leaf sheaths of rice plants artificially inoculated in the greenhouse. Ascocarps have not been produced in culture.

Another distinctive group in Euhelminthosporium comprises species such as *H. turcicum*, *H. rostratum* Drechs., *H. monoceras* Drechs., *H. micropus* Drechs., and *H. frumentacei* Mitra, in all of which the conidia are provided with a protuberant hilum. The present study demonstrates that the perfect stage of one of these species, *H. turcicum*, may be placed in the genus *Trichometasphaeria*. A perfect stage closely resembling *T. turcica* has also been found in a second species of this group, *H. rostratum* (16). It is tempting to predict a correlation between the production of perfect stages of the *Trichometasphaeria* type and the formation of conidia in which a protuberant hilum is present. However, Drechsler (10) stated that he had found immature perithecia of the *Cochliobolus* type in cultures of *H. micropus*, and I have on one occasion obtained mature *Cochliobolus* perithecia in matings of *H. micropus* isolates. Discovery of perfect stages in additional species and a study of conidium formation in the group represented by *H. turcicum* and *H. rostratum* will be necessary to determine whether any correlation exists between conidial characteristics and type of perfect stage in this group.

Demonstration of heterothallism in *Trichometasphaeria turcica* is of especial interest since this phenomenon has rarely been reported in members of the Loculoascomycetes. The paucity of data on this point may simply reflect inadequate study arising from the difficulty of producing mature ascocarps in culture in the majority of these species. Heterothallism has been demonstrated previously in *Venturia inaequalis* (Cke.) Wint. (14), a species that has been the subject of extensive genetic study (1).

Although improved methods would be desirable, the cultural procedures outlined herein furnished a basis for the discovery of perfect stages in additional species of *Helminthosporium*. Knowledge of these perfect stages would make possible a more natural classification of the pathogens of grasses and cereals now included in the form genus *Helminthosporium*. Such investigations would also furnish materials for genetic studies in this important group of pathogens.

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PHYTOPATHOLOGICAL NOTES

Isolation of a Sporulating Strain of Cercospora kikuchii by Selective Sub-Culturing. JOHN P. JONES.[1] *Cercospora kikuchii* T. Matsu. & Tomoyasu sporulates sparsely in culture.[2, 3] Murakishi[2] tried 15 different types of media in an effort to induce sporulation, but was unsuccessful. Kilpatrick and Johnson,[3] however, obtained moderate sporulation in culture by streaking spores on a carrot leaf decoction agar. A different approach to the problem was used by Calpouzos,[4] with *Cercospora musae* Zimm. He secured sporulating strains of *C. musae* by selective sub-culturing. By careful examination of young colonies under a dissecting microscope, Calpouzos detected tiny sporulating areas, which he transferred to fresh media. The resulting colonies were examined in turn and sections of sporulating areas transferred. After a number of such transfers, a pure sporulating strain was secured.

An attempt was made to obtain a sporulating strain of *C. kikuchii* by following the method of Calpouzos. A culture of *C. kikuchii* isolated by spore transfer from a soybean stem was found to have 2 sporulating sectors, each about 4 mm in diameter. Portions of these sectors were transferred to fresh PDA in Petri plates, which were then incubated at room temperature on a table exposed to daylight. The resulting colonies were examined daily until sporulating areas appeared (4–6 weeks after transfer), when small portions (about 2 × 2 mm) were transferred to fresh media, and the process was repeated through a series of 7 sub-cultures. The final group of transfers was made 10 months after the first. About 20–25 portions of the sporulating sectors were transferred at each sub-culturing.

The sporulating strain obtained by this process produced abundant spores evenly distributed over the surface of the colony. Sporulation occurred in 5–7 days in colonies produced from transferred mycelium or spores. The type of sporulation in culture differed somewhat from that on host tissue, in that the geniculated conidiophores arose singly from mycelial strands and did not occur in fasicles that arose from stromata.

[1] Pathologist, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, and Mississippi Agricultural Experiment Station, cooperating.

[2] Murakishi, H. H. 1951. Purple seed stain of soybean. Phytopathology 41: 305-318.

[3] Kilpatrick, R. A., and H. W. Johnson. 1956. Sporulation of Cercospora species on carrot leaf decoction agar. Phytopathology 46: 180-181.

[4] Calpouzos, L. 1955. Studies on the Sigatoka disease of bananas and its fungus pathogen. Atkins Garden and Research Laboratory, Cienfuegos, Cuba. 79 p.

On steamed soybean stems, however, the conidiophores occurred in fasicles as on naturally infected soybean stems. The sporulating isolate produced dark gray mycelium with a uniform felt-like texture. It produced abundant dark-purple pigment on PDA, bean pod, and nutrient dextrose agars, but a lesser amount on cornmeal agar.

A series of 75 single-spore isolations were made from the sporulating strain to observe if it was stable. The resulting 62 identical sporulating colonies indicate stability. The strain has remained uniform to date.

This method offers a means of obtaining abundant, consistent conidial production by *C. kikuchii*.—Delta Branch Mississippi Agricultural Experiment Station, Stoneville, Mississippi.

Effect of Maleic Hydrazide, Thiourea, and 2,4-Dinitrophenol on Resistance to Flax Wilt[1]. P. N. NAIR.[2] Maleic hydrazide (MH), thiourea, and 2,4-dinitrophenol (DNP) are known to inhibit phosphorylation of certain steps in the respiratory cycle in higher plants.[4, 5] It is also known that when these substances are introduced into the transpiration stream, tomato cuttings lose their resistance to wilt caused by *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyd. & Hans.[4, 5] The same chemicals were used to determine their effects on the resistance of 2 flax varieties, B5128 and Redwood, to wilt caused by *Fusarium oxysporum* f. *lini* (Bolley) Snyd. & Hans.

In the first of 2 greenhouse experiments, the plants were grown in wax-coated paper cups, conical in shape and open at both ends, filled with a 1:1 mixture of steam-sterilized soil and vermiculite. The wider ends of the cups were closed with cotton plugs and placed in sterile petri dishes (covers removed) that were half filled with the same soil-vermiculite medium. Three cups for each treatment were used, with 5 seedlings of each variety per cup. The soil-vermiculite medium was infested with a spore suspension of *F. oxysporum* f. *lini*. The infested and noninfested sets were placed side by side in randomized blocks. The greenhouse temperature ranged from 65° to 75°F. When the seedlings were about 2–3 in. tall, 50 ml of each chemical was poured on the soil of each cup and dish. In the second greenhouse experiment, 40–50 plants, in each of 3 replicates, were grown in 4-in. pots, and 100 ml of the chemical was added to each pot.

TABLE 1.—*Percentage of plants wilted in 2 varieties of flax inoculated with Fusarium oxysporum f. lini and treated with maleic hydrazide, thiourea, or 2,4-dinitrophenol at 3 concentrations*[a]

Chemical	Molar concentration	Percentage of plants wilted[b] in indicated variety	
		B5128	Redwood
None		29	9
Maleic hydrazide	2×10^{-4}	46	9
	5×10^{-4}	78	15
	10^{-3}	80	22
Thiourea	2×10^{-4}	27	16
	5×10^{-4}	52	37
	10^{-3}	84	39
2,4-Dinitrophenol	10^{-5}	87	40
	2×10^{-5}	89	41
	5×10^{-5}	99	46

[a] Plants were grown in infested soil, and the chemicals were added to the soil when seedlings were 2–3 in. tall.

[b] Average of 3 replicates, 40–50 plants per replicate. There was no wilt in plants not inoculated.

TABLE 2.—*Dry weights of Fusarium oxysporum f. lini cultured on media containing maleic hydrazide, thiourea, or 2,4-dinitrophenol at 3 concentrations.*

Chemical	Molar concentration	Mean weight (mg)[a]
None		66
Maleic hydrazide	2×10^{-4}	141
	5×10^{-4}	117
	10^{-3}	113
Thiourea	2×10^{-4}	168
	5×10^{-4}	520
	10^{-3}	462
2,4-Dinitrophenol	10^{-5}	280
	2×10^{-5}	73
	5×10^{-5}	73
H.S.D. (p=0.01)[b]		24.3
L.S.D. (p=0.01)[c]		16.1

[a] Based on dry weights of fungus, 3 flasks per treatment.

[b] Honestly significant differences can be used to test all possible comparisons. It will be conservative for all but the largest difference.[3]

[c] Least significant difference is valid only for comparing adjacent means when the means are ranked according to magnitude.[3]

Three concentrations of each chemical were used. MH and thiourea were applied at the concentrations of 2×10^{-4}, 5×10^{-4}, and 10^{-3} M; DNP was used at 10^{-5}, 2×10^{-5}, and 5×10^{-5} M.

The results obtained in both experiments are given in Table 1. The noninoculated plants were all free of symptoms. The percentages of plants wilted among treated inoculated plants were greater than those among nontreated inoculated plants. Numbers of wilted plants increased with an increase in concentration of the chemicals. Although all 3 chemicals de-

[1] Paper No. 3713, Scientific Journal Series, Minnesota Agricultural Experiment Station.

A portion of a thesis submitted to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Formerly Research Assistant, Department of Plant Pathology and Botany, University of Minnesota.

[3] Federer, W. T. 1955. Experimental design. Macmillan Co., New York. 591 p.

[4] Gothoskar, S. S., R. P. Scheffer, M. A. Stahmann, and J. C. Walker. 1955. Further studies on the nature of Fusarium resistance in tomato. Phytopathology 45: 303-307.

[5] Waggoner, P. E., and A. E. Dimond. 1952. Effect of stunting agents, Fusarium lycopersici and maleic hydrazide, upon phosphorus distribution in tomato. (Abstr.) Phytopathology 42: 22.

creased wilt resistance, DNP decreased it most. The percentages of plants wilted when treated with DNP at all 3 concentrations and with MH and thiourea at the highest concentrations were significantly higher, statistically, than were those at other treatments (including the nontreated controls). Redwood did not lose its wilt resistance completely in any of the treatments.

To determine whether the increase in the percentage of wilted plants was brought about by the stimulatory effect of the chemicals on the pathogen, *F. oxysporum* f. *lini* was cultured on Pfeffer's modified nutrient solution containing 1.5 per cent glucose to which MH, DNP, or thiourea was added. Each medium was adjusted to pH 6 ± 0.1 and buffered with phosphate buffer. The fungus grew on a shaker for 6 days at 27°–30°C.

All of the chemicals at the concentrations used stimulated growth of the pathogen (Table 2). The stimulatory effect decreased with an increase in concentration of MH or DNP. The higher concentrations of thiourea had a greater stimulatory effect than did the lower concentrations.

The amount of growth of the pathogen on media containing the various chemicals was not correlated with the severity of wilt in plants treated with the respective chemicals. Therefore, the chemicals apparently did not increase the number of wilted plants solely by stimulating growth of the pathogen, if growth of the pathogen on culture media containing the chemicals is accepted as a sufficient criterion of the effect of the chemicals on growth. It is probable that the chemicals affected the host or the host-pathogen relationship to make the plant more susceptible to wilt.—Department of Plant Pathology and Botany, Institute of Agriculture, University of Minnesota, St. Paul 1, Minnesota.

LISTING OF BOOKS

PHYTOPATHOLOGY will, from time to time as space becomes available, list titles of books on pathological subjects. A brief description of the contents of the books will be included.

AMUNDSEN, V., ed. 1957. **Yearbook.** Royal Veterinary and Agricultural College, Copenhagen, Denmark. 141 p.

This Yearbook contains a variety of contributions representing the current activities of its institution. The language used is English. Among the contributions are 2 items that may be of special interest to American phytopathologists. The first is an index of the Yearbook series. This index, compiled by Astri Fossum, covers the years 1917 through 1956 and is arranged by subjects, with individual contributions in each field arranged alphabetically by authors. The second is an extensive treatise entitled *Correlations and interrelationships in viruses and in organisms. II. The principles of the natural periodical system of plant and animal infecting viruses.* This paper by H. P. Hansen occupies pages 31 through 66 in the 1957 Yearbook and constitutes Contribution No. 48 from the Department of Plant Pathology, Virological Laboratory, Royal Veterinary and Agricultural College, Copenhagen. All those who are concerned with methods of naming and classifying viruses will want to examine this publication, which attempts to integrate the classification of viruses pathogenic to plants and animals.

WAGER, V. A. 1956. **Common diseases of vegetables in South Africa.** Department of Agriculture, Union of South Africa, Pretoria. 175 p. Price 12 s.

Some 78 diseases of vegetables are illustrated, and briefly annotated, in this small book that was planned for quick and easy reference. Except for a few pages devoted to general remarks and information on seed and tuber treatment, each odd-numbered page contains photographs of symptoms, often accompanied by drawings of the fruiting structures or other identifying features of the causal agent. On the page opposite the illustrations of a particular disease, very brief remarks are given, in both English and Dutch, on diagnostic symptoms, etiology, and control.

BAKER, K. F., ed., AND 9 OTHERS. 1957. **The U. C. system for producing healthy container-grown plants.** California Agricultural Experiment Station Manual 23. 332 p., 136 fig. Price $1.00, from Agricultural Publications, University of California, Berkeley 4, California.

A unified system is presented for commercial production of healthy plants in flats, pots, benchs, beds, and other containers. Emphasis is placed on the principles behind the procedures discussed for eliminating

soil pathogens at source through the use of clean soil, clean stock, and sanitation. The 17 sections, written by 10 specialists, cover the dynamics of disease (including pathogen variability and inoculum potential), salinity problems, soil mixes, treatment of soil and containers by heat and chemicals, pathogen-free planting stock, beneficial soil microorganisms, grower experience with the system, and mechanization. A general summary, bibliography, glossary, and index are provided.

DUDDINGTON, C. L. 1957. **The friendly fungi.** The Macmillan Company, 60 Fifth Avenue, New York 11, N. Y. 188 p. Price $4.50.

As suggested by its subtitle, *A New Approach to the Eelworm Problem*, this book deals with the predacious fungi that capture and kill nematodes. It is written in non-technical language, but it is sufficiently comprehensive and detailed to serve as an introduction to the study of this group of fungi. The first 2 chapters deal with the nematodes themselves. The next 4 chapters discuss the various fungi that attack nematodes, with emphasis on the several mechanisms by which they trap and kill nematodes. The last 3 chapters describe some of the attempts that have been made to use such fungi for the control of nematodes in crops. The appendix contains a short account of the techniques commonly used in the study of predacious fungi.

RATAJ, K. 1957. **Škodlivá činitelé přadnych rostlin.** Prameny Literatury. Czechoslovak Academy of Agricultural Sciences, Praha. Vol. 2, No. 1-2, 123 p.

The author gives a comprehensive annotated bibliography of the diseases and other factors that are detrimental to the fiber plants grown in Czechoslovakia. Most of the citations concern flax and hemp. The abstracts are in Czech and arranged in sections according to the harmful factor. The general introduction, the title, a short explanatory paragraph on each section, and the table of contents are in Russian, German, English, and Czech. The work is a convenient guide to the literature on the diseases and pests of flax and hemp, particularly to papers published in eastern Europe.